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Innovative novel immunotherapies for the treatment of glioblastoma multiforme

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INNOVATIVE NOVEL IMMUNOTHERAPIES FOR THE TREATMENT OF GLIOBLASTOMA MULTIFORME

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For my husband” Emad “and my kids “Adel, Hana, and Nor”

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ABSTRACT

Salem, Salma. M.S., Purdue University, December 2016. Innovative Novel Imuno Therapies for the Treatment of Glioblastoma Multiforme. Major Professor: Kari Clase.

Glioblastoma Multiforme GBM is a very aggressive type of malignant brain tumors that affects peoples' lives. The diffusive, infiltrative, and metastatic behaviour of GBM is the major reason for the disease recurrence. The morphological and immunohistological characteristics of Central Nervous System (CNS) tumors including GBM are heterogeneous. GBM is either primary (de novo) or secondary to low-grade astrocytomas.

Current treatment options include surgery, radiotherapy, and temozolomide chemotherapy have not achieved any improvement in success rates over the past decades. The survival time reached by GBM patients was approximately 12 months only after being treated with radiotherapy alone without temozolomide. However, the median survival time has been estimated as 14.6 months in patients who received the combined treatment of radiotherapy and chemotherapy with temozolomide. Temozolomide is an alkylating agent that exhibit antitumor activity and prescribed as a single agent for the treatment of recurrent glioma. It diminishes the O^6-methylguanine-DNA methyltransferase (MGMT) enzyme, which is responsible for DNA repair. GBM

Patientswith activated O6-alkylguanine transferase AGT enzyme were reported to develop resistant to temozolomide.

Targeted therapies are other options for GBM patients. Bevacizumab blocks the effect of Human Vascular Endothelial Growth Factor (VEGF) and inhibits tumor vascular growth. Regulatory concerns about clinical trials conducted with bevacizumab including trial design problems have been raised.

Targeting the immune system will identify successful treatments for GBM with significant clinical benefits. The use of active immunotherapy to increase the native immune response or passive immunotherapy to target the tumor cells in GBM patients are under investigation. Dendritic Cells DCs are the most potent antigen presenting APCs in the immune system. DCs have the ability to stimulate the native T cells and induce primary immune responses and peripheral immunological tolerance through capturing, processing neoantigens, which are formed and released by oncogenesis, and presenting the captured antigens on Histocompatibility Complex I and II (MHCI and MHCII) molecules to T cells. After that T cell responses against the cancer-specific antigens are primed and activated. T cells infiltrate the tumor bed, specifically recognize, bind to, and kill their target cancer cell. The sources of antigen that have been used in DC immunotherapy include exogenous MHC-restricted peptides, acid-eluted tumor peptides, tumor RNA and cDNA, viral vectors, apoptotic tumor cells, tumor cell lysate, and whole glioma cells. Clinical trials showed that treating GBM patients with surgery, TMZ, and Dendritic Cell DC vaccines was safe and achieved meaningful clinical outcomes.

The purpose of this research is to investigate the clinical outcomes of DC vaccines for the treatment of GBM patients through the published data on the rapidly growing field

of clinical trials. Furthermore, this research focuses on investigating immune responses that are related to the most beneficial clinical outcomes to identify new prognostic biomarkers and therapeutic targets.

This research's findings have showed evidence that many different variables were associated with different cancer specific immune responses and clinical outcomes. These variables include loading DC vaccines with tumor material or Tumor Associated Antigens TAAs, and combining DC vaccines with different pre and post vaccination treatment strategies. DC vaccine pulsed with specific synthetic antigens have achieved more beneficial clinical outcome than DC vaccines loaded with tumor material. Pre-vaccination treatment strategy with TMZ has increased the Overall Survival and the Progression Free Survival of GBM patients. Although, pre-vaccination treatment strategy with TMZ+RT has achieved significant improvement of Overall Survival, there was no improvement of the Progression Free Survival associated with the strategy.

CHAPTER 1. INTRODUCTION

This chapter introduces the overview of Glioblastoma Multiforme (GBM) research work. This chapter provides a statement of the problem in GBM cancer studies, research question, scope, significance, definitions of key terms, assumptions, limitations, and delimitations of this research study.

1.1 Statement of the Problem

Glioblastoma Multiforme GBM is a very aggressive type of malignant brain tumors that affects peoples' lives. GBM has the ability to escape immune system surveillance and causes major mutations in cell signaling and growth. Current treatment options including surgery, radiotherapy, and temozolomide chemotherapy have not achieved any improvement in success rates over the past decades. Targeting the immune system will identify successful treatments for GBM with significant clinical benefits. The purpose of this research is to investigate innovative immunotherapies for GBM by using data published on the rapidly growing field of clinical trials.

1.2 Research Question

Do innovative immunotherapies for Glioblastoma Multiforme have the ability to increase the activation of patients' immune system and successfully treat the disease?

1.3 Scope

The scope of this research is to investigate the innovative immunotherapies for the treatment of Glioblastoma Multiforme (GBM) using available published literature and various data analysis techniques. This study is conducted to explore the clinical outcomes of Dendritic Cell vaccines; which are currently under investigation for the treatment of GBM. The study is exploring the efficiency of the treatment combination of immunotherapy, chemotherapy, following surgical resection of the tumor mass. Furthermore, the study focuses on identifying immune responses, prognostic biomarkers, and new therapeutic targets for GBM.

1.4 Significance

The treatment options of Glioblastoma Multiforme, including surgical removal, radiation therapy, and cytotoxic chemotherapy, are very aggressive as well as they do not achieve any improvement in the disease state. This research will use data published on the rapidly growing field of clinical trials to provide in depth knowledge about GBM and host factors that are related to immune system interactions.

The research investigates innovative immunotherapies, which present a potential solution to treat Glioblastoma Multiforme. The result of this work will be a seed for future research on identifying a new treatment with significant clinical benefits that is capable of handling the obstacles that are facing the development of glioblastoma immunotherapies.

1.5 Definitions of Key Terms

Apoptosis is a genetically encoded cell death program which is well known by the corresponding morphologic and biochemical changes (Fisher, 1994).

Biomarkers are measureable quantities of biologic homeostasis that are used to differentiate between normal and abnormal and can be detected using recent technological advances (Dalton & Friend, 2006).

Brain metastases are brain tumors that originate in specific tissue of origin outside the brain and metastasize to the brain which resemble more than 50% of all brain tumors in adults (Jacobs et al., 2009).

Carcinogen is a chemical, physical or viral stimuli which directly induces cancer by formation of DNA adducts and initiation of various genetic mutations (Higginson, 1987; Herbst, Heymach, & Lippman, 2008).

$CD25^+$ $CD4^+$ are suppressor Tregs lymphocytes which make the immunity system unresponsive to self-constituents, establish what is known as self-tolerance, and maintain a negative control of pathological and physiological immune responses. (Sakaguchi, 2004).

Dendritic cells (DCs) are antigen-presenting cells, which act by capturing and transferring information from the surrounding environment to the cells of the adaptive immune system. They can induce primary immune responses, immunological tolerance, and regulate T cell–mediated immune response (Banchereau et al., 2000).

Immune system is the body system, which recognize foreign invaders and eliminate them. It has the ability to distinguish between self- and non-self-constituents (or antigens) (Thomas, Ernstoff, & Fadul, 2012). The immune system includes two major branches: the innate and the adaptive immune systems (Kanaly, Ding, Heimberger, & Sampson, 2010).

Oncogene is mutated gene which is resulted from different carcinogens and causes intensive cell growth that proliferates into cancer cells (Herbst et al., 2008).

Temozolomide (TMZ) is DNA alkylating agent which exhibits antitumor activity and is used for the treatment of Glioblastoma Multiforme (Stupp et al., 2005).

1.6 Assumptions

The assumptions of this thesis will include:

- The sources of data are trusted.
- Data analysis will be accurate and no data will be missed.

1.7 Limitations

The following limitations are inherent to the pursuit of this study:

- The research will investigate the innovative dendritic cell vaccines for the treatment of Glioblastoma Multiforme (GBM).

1.8 Delimitations

The following delimitations are inherent to the pursuit of this study:

- This study will not investigate the efficacy of the chemotherapy or the radiotherapy treatments.
- This study will not investigate the adaptive immunotherapies.

1.9 Summary

This chapter has provided an overview of the research study, including statement of purpose, research question, scope, and significance, definitions of key terms, assumptions, limitations, and delimitations.

CHAPTER 2. LITERATURE REVIEW

This chapter presents an overview of Glioblastoma Multiforme (GBM), Epidemiology, Metastases, cancer stem cell hypothesis, treatment options “Radiotherapy, Bevacizumab, and Temozolomide”, immune reactions in healthy brain and in Glioblastoma patients, immunotherapy for the treatment of GBM, adoptive immunotherapy, active immunotherapy, and nanotechnology in immunotherapy for the treatment of GBM. It also introduces DC development, diversification, maturation, and function, tumor associated antigens TAA, and end points and evidences of therapeutic activity.

2.1 Glioblastoma Multiforme

Glioblastoma Multiforme (GBM) is a brain tumor with severe manifestations of anaplasia and dedifferentiation of glia, which is representing about 50 % of all gliomas. Glioblastoma is the most aggressive, complex, and common human brain tumor type, which is known as grade four gliomas (Holland, 2000). Morphological and immunological features show that the proliferation of Glioblastoma is accompanied by immunological reaction against tumor-specific antibodies. It is the most malignant primary intracranial neoplasm, which is identified by a gross and microscopic morphology and affects mostly the older age populations. The disease can rarely affect

ages under 40 years, although the peak age incidence lies between 48 and 55 years (Jellinger, 1978).

CNS tumors such as Glioblastoma Multiforme are caused by genetic mutation that results in a progressive neoplastic transformation of differentiated cells (Jellinger, 1978). The genetic mutation is characterized by several deletions, amplifications, and point mutations that is followed by activation of signal transduction pathways, inhibition of tyrosine kinase receptors, and disturbance in cell-cycle arrest pathways either by INK4a-ARF gene loss or tumor suppressor p53 gene mutations (Holland, 2000).

2.2 Epidemiology

Central nervous system (CNS) primary tumors are affecting approximately 18.71 per 100,000 persons per year (States & others, 2010). Primary brain tumors are the main cause of deaths in cancer patients and it represents about 2.3 % of deaths in cancer patients in Europe and North America (Canadian Cancer Society's Steering Committee, 2010).

Glioma is the most common primary brain tumor with the incidence percent of 32 % of CNS tumors and 80 % of malignant CNS tumors as shown in Figure. 2.1 (Agnihotri et al., 2013).

Glioblastomas are the most malignant glioma and the main type of astrocytoma with an incidence percent of about 54 % of the astrocytic tumors. The incidence ratio of Glioblastomas is 1.58:1 in men and women and 2:1 in Caucasians and African-Americans respectively (States & others, 2010). Malignant gliomas are thought to be related to family history in about 5 % of patients who usually experience a rare genetic

syndrome such as neurofibromatosis types 1 and 2, and Li-Fraumeni syndrome (Farrell & Plotkin, 2007).

2.3 Metastases

Metastasis is an invasive property that arises from interactions between cancer cells and their microenvironment. This action is mostly due to the loss of the cell-cell adhesion that is controlled by E-cadherin. The inactivation of E-cadherin originates from inactive protein, gene silencing or the overexpression of the growth factor receptors.

There are also a number of genes which are responsible for genetic and epigenetic mutations in cancer cells and are supported by micro environmental changes to initiate metastatic behaviour of tumors (Chiang & Massagué, 2008). These genes are divided into three groups: initiation, progression, and virulence genes (Nguyen & Massagué, 2007). Genes that are responsible for the progression of metastasis make the cancer cell capable of traveling successfully between different points until it reaches the distant site that is identified by those genes. Genes that cause metastatic initiation are operating in the primary tumor site and the distant metastatic site. This classification of the genes that are responsible for cancer metastasis and their functions is important to provide a multidimensional explanation of metastasis and is crucial to establish several anti-metastatic strategies (Chiang & Massagué, 2008).

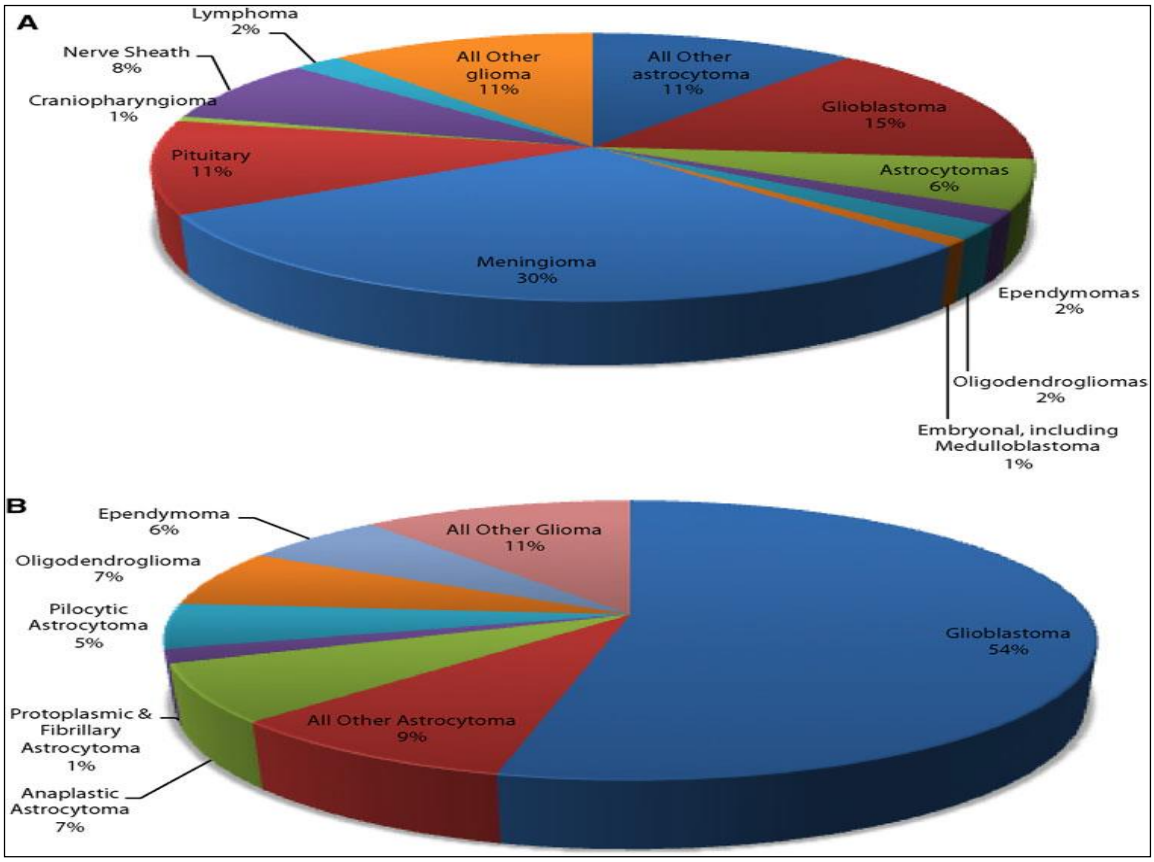

Figure 2.1 Primary brain tumor epidemiology. A shows the percent distribution of all a primary brain and CNS by histology. B shows the percent of primary brain gliomas in the United States (2004-2006) (Agnihotri et al., 2013).

Glioblastomas or grade four gliomas are more proliferative, infiltrative, and invasive in nature than grade one, two, and three astrocytoma (Kleihues & Sobin, 2000). This diffusive and infiltrative nature of glioblastoma is the major reason for the surgical incurability and disease recurrence. The malignant cells of Glioblastoma disseminate from the primary tumor site and migrate in specific routes that depend on the structure of the brain and the connected extra cellular matrix (Agnihotri et al., 2013). Glioblastomas migrate commonly through dispersing in the tracks of the white matter, the basal lamina of the brain blood vessels, or in between the glia limitans and the pia mater (Bellail, Hunter, Brat, Tan, & Van Meir, 2004).

2.4 Cancer Stem Cell Hypothesis

The original cell of GBM that is predicted to develop neoplastic lesions to initiate cancer follows three possible assumptions. The first assumption is the ability of a dedifferentiated mature glia to develop abnormal stem-cell like properties through epigenetic lesions that is initiated by mutation. The second assumption is that restricted neural progenitors with a limited self-renewal property can develop mutations and also gain stem-cell like properties. The third assumption is that adult neural stem cells (NSCs), which are normally proliferate and differentiate is capable of developing mutations and tumor formation (Dirks, 2008; Dirks, 2001; Stiles & Rowitch, 2008). Glioblastoma arises from cells that are able to obtain stem-cell like properties, grow abnormally, and initiate tumor formation. This concept is the base of the cancer stem cell hypothesis, which is shown in Figure 2.2 (Agnihotri et al., 2013). Normal cells of the central nervous system differentiate and central nervous system tumor is initiated. Neural stem cells are forming neural and glial progenitors, which differentiate into the principle cell types of the central nervous system neurons, oligodendrocytes and astrocytes. Tumor initiating cells (BTICs) are thought to come from terminally differentiated cells as shown in Figure 2.2 and developed from the transformation of neural stem cells or neural progenitors.

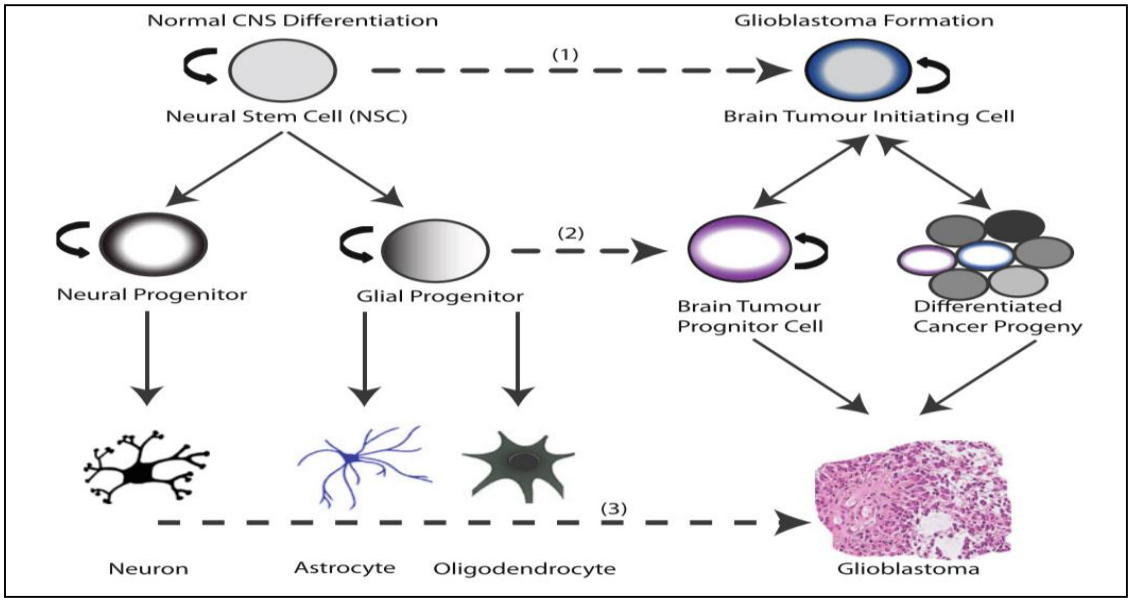

Figure 2.2 The original cells of Glioblastoma Multiforme (Agnihotri et al., 2013).

Central nervous system (CNS) tumors are classified based on their morphological and immunohistological characteristics which outline the predominant cell type. GBM can be classified into two main subgroups with different genetic pathways. Primary or de novo GBM occurs mostly in older patients with no in advance occurrence of low-grade astrosytomas. However, secondary GBM occurs in young patients and originates from low-grade astrocytomas as shown in figure 2.3 (Louis et al., 2007).

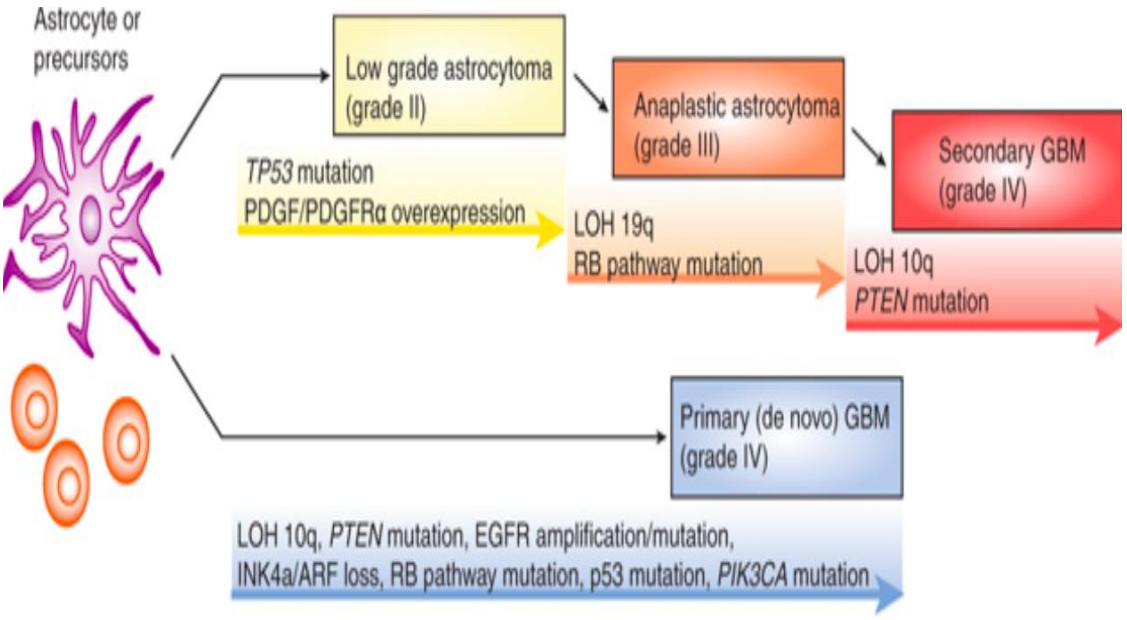

Figure 2.3 GBM subtypes with different genetic pathways (Endersby & Baker, 2008)

2.5 Treatment Options for Glioblastoma Multiforme

The current standard of treatment options of Glioblastoma have not changed over the last decades. The first line of treatment is the surgical resection of the tumor, followed by radiotherapy. In the United States, a nitrosourea drug (carmustine) is usually recommended ; (Walker, Laherty, Tomlinson, Chuah, & Schmidt, 2008).

Complete surgical resection of tumor mass is impossible due to the topographically diffusive nature of the disease and the large location variability of the tumor cells within the brain. This behavior results in the diffusion of the tumor cells within large distances, particularly into the brain vital regions that are crucial for patient's survival. Recurrence of the tumor can occur at the surgical margin and other sites after a total resection of Glioblastoma (Holland, 2000).

2.6 Radiotherapy

Radiotherapy and chemotherapy are following the surgical resection of the tumor and achieve an expected mean survival that lies between two months and one year (Jelsma & Bucy, 1967). There is a great improvement in survival time resulting from the combination treatment of radiotherapy and chemotherapy using temozolomide with a median survival time of 14.6 months (Dieckmann, 2010). Modern radiotherapy aims to increase the dose that is delivered to the tumor region, decrease the dose that is delivered to the normal brain tissue, and avoid local necrosis (Stupp et al., 2005). The combined treatment of radiotherapy and chemotherapy with temozolomide in Glioblastoma has achieved a median survival of 14.6 months; however, the survival time was 12 months only without temozolomide. The image-based conformal radiotherapy achieved two years, which is considered crucial before and after surgical resection (Stupp, 2006; Stupp et al., 2002).

The standard technique of radiotherapy is based on three-dimensional conformal processes that depend on magnetic resonance imaging and x-ray computed tomography fused data sets. There is an optimal advantage over the use of these data sets in the pre-operative and the post-operative stages. The pre-operative gross tumour volume measurement achieves a significant advantage in the postoperative structures evaluation. Follow-up is necessary in order to increase the efficiency of the radiotherapy techniques and to evaluate the post-treatment improvement (Dieckmann, 2010).

2.7 Bevacizumab

Bevacizumab is a recombinant humanized monoclonal IgG1 antibody, which targets human vascular endothelial growth factor (VEGF) and prevents the binding of VEGF to endothelial cells receptors. Blocking the effect of VEGF leads to the inhibition of tumor vascular growth (Cohen, Shen, Keegan, & Pazdur, 2009). Glioblastoma cells produce enormous amounts of VEGF in situ, and the inhibition of these factors hinders the growth of glioma xenografts in immune-deficient mice (Stefanik et al., 2001). Bevacizumab is an antiangiogenic compound which inhibit the ability of the malignant gliomas to generate tumor-associated blood vessels (Takahashi et al., 1992; Maxwell et al., 1991).

A single-arm clinical trial was conducted to evaluate the biological activity and safety of bevacizumab in patients with recurrent high-grade gliomas. Dosing strategy contained an intravenous infusion of 10 mg/kg bevacizumab every 2 weeks on a 4-week cycle until the disease was progressed or major toxic effects were observed. Patients with a confirmed brain malignant glioma and who were ≥ 18 years of age with disease progression after receiving radiotherapy were enrolled in the trial. Patients with identified bevacizumab risk factors and acute intracranial/ intratumoral bleeding were excluded from the trial (Cohen et al., 2009). The evaluation of tumor response based on the modified World Health Organization WHO response evaluation criteria (Macdonald, Cascino, Schold, & Cairncross, 1990).

Another study combined the two-week interval of intravenous infusion of bevacizumab (10 mg/kg) and irinotecan (340 mg/m2) until disease progression, major toxic side effects, or a maximum of 104 weeks of treatment. The clinical trials were

designed to estimate the biological activity and safety of bevacizumab alone or combined with irinotecan in patients with recurrent glioblastoma. The pre-planned activity endpoints were the 6-month Progression-free survival PFS rate and objective response rate. The clinical trial was conducted through June 30, 2006 to September 15, 2007 and the drug was approved by the U.S. FDA on May 5, 2009.

Pediatric phase I clinical trial of bevacizumab was conducted on 19 pediatric patients with solid tumors. The aim of the study was identifying the maximum-tolerated dose (MTD) and estimating the dose-limiting toxicities (DLTs), pharmacokinetics, and efficacy. Bevacizumab was effective in the treatment of pediatric patients, with common side effects of infusion reaction, rash, mucositis, and proteinuria. BV achieved an acceptable toxicity profile when administered at doses of 5, 10, or 15 mg/kg every 2 weeks. No dose-limiting toxicities (DLTs) which require discontinuation of the treatment were observed. A single pediatric patient experienced a longstanding rise in both diastolic and systolic blood pressure BP after receiving bevacizumab treatment. BP elevation usually occurs in the majority of adult and pediatric patients who are treated with the drug. This side effect did not meet the Common Terminology Criteria for Adverse Events (CTCAEv3) pediatric-specific criteria for hypertension except in a one pediatric patient. No effects on open epiphyses were observed during the limited time of the clinical trial. Luteinizing hormone (LH) and follicle-stimulating hormone FSH levels were elevated in two of three postmenarchal females which is consistent with inhibition of the biological function of the ovary. Pharmacokinetic studies showed that the bioavailability of the drug was proportional to the dose (Bender et al., 2008). There were a major differences in drug disposition in children, which were also observed in adult patients (Gordon et al.,

2001; Gaudreault et al., 2004; Hsei et al., 2001;Lu, Gaudreault, Novotny, Lum, & Bruno, 2004). A minimum efficacy was observed after conducting a phase II study of both bevacizumab and irinotecan on pediatric patients with recurrent malignant glioma (MG) and intrinsic brainstem glioma (BSG) (Gururangan et al., 2010).

Regulatory concerns about this trial contained trial design problems, the lack of an identified endpoint, and the lack of an appropriate method to confirm the results. Evaluating the efficacy of bevacizumab in the combination therapy with irinotecan is very difficult. There is no standard reference to compare with the 6-month PFS and the overall survival rate endpoint. Using a historically controlled groups in the comparison with PFS or overall survival is unrealistic because they cannot be considered as a proof of the biological activity of the drug. The difference between the clinically observed responses and the true measurements of tumor size using the magnetic resonance imaging MRI has encountered this trial (Cohen et al., 2009).

The European drug regulatory agency, EMEA (European Medicines Agency) refused the approval of bevacizumab for recurrent GBM for the reason that the benefits of bevacizumab in the treatment of newly diagnosed GBM patients did not overweigh the risks (Refusal of a change to the marketing authorization for Avastin (bevacizumab),2014). The most common side effects of Bevacizumab include; fatigue, headache, and hypertension. Patients have experienced grade 3 or more toxic side effects which included hypertension, convulsion, arterial thromboembolism, venous thromboembolism, wound-healing complications, and intracranial hemorrhage (Friedman et al., 2009). The Avastin package insert has safety information about: arterial

thromboembolic events and infusion reactions, gastrointestinal perforation, reversible posterior leukoencephalopathy syndrome (RPLS), nasal septum perforation, and non-gastrointestinal fistula formation (FDA Briefing Document Oncology Drug Advisory Committee Meeting, 2010).

2.8 Temozolomide

Temozolomide is an alkylating agent that is prescribed as a single agent for the treatment of recurrent glioma. The drug is administered orally and exhibit antitumor activity (Newlands, Stevens, Wedge, Wheelhouse, & Brock, 1997; W. K. A. Yung et al., 2000).The dosing regimen consists of 150 to 200 mg for the square meter of body-surface area to be administered once daily for 5 days of every 28-day cycle (Stupp et al., 2005). Another dosing regimen consists of 75 mg for the square meter to be administered once daily during seven weeks (Brock et al., 1998). Temozolomide diminishes the O^6-methylguanine-DNA methyltransferase (MGMT) enzyme which is responsible for the repair of the DNA (Tolcher et al., 2003). The decrease in the O^6-methylguanine-DNA methyltransferase enzyme in tumor site is found to increase the survival of the Glioblastoma patients who are receiving nitrosourea-based adjuvant chemotherapy (Esteller et al., 2000; Hegi et al., 2004).

Temozolomide is a prodrug with a small size (194 Da) which is optimally absorbed in the small intestine and penetrate the blood-brain barrier. It undergoes spontaneous hydrolysis in the cells and converts to a strong methylating agent MTIC. MTIC methylates the guanine as well as several base nucleobases. Nicks in the DNA are

formed and apoptosis occur as a result of the failure of the cellular repair mechanisms to repair the methylated base (Wesolowski, Rajdev, & Mukherji, 2010).

A randomized phase III trial showed that temozolomide had increased the median survival and achieved a 2-year survival time after the drug was added to the standard postoperative radiotherapy. 573 patients from 85 institutions in 15 countries were randomly enrolled in the trial including 286 patients have received radiotherapy alone and 287 patients have received temozolomide combined with radiotherapy. Longer survival was observed in patients (with a methylated and un-methylated MGMT promoter) who have received the combined therapy of temozolomide and radiotherapy than in patients who have received radiotherapy alone (Stupp et al., 2009).

There are other three meaningful studies involved patients with malignant glioma who have received previous treatment but encountered disease progression. One major study had investigated the effects of Temodal in 138 patients with Glioblastoma Multiforme. Another study compared Temodal with procarbazine in 225 GBM patients. The final study investigated the safety and effectiveness of Temodal in the treatment of 162 patients with anaplastic astrocytoma who were in their first relapse. Effectiveness was evaluated on the basis of patients' survival time and how long did it take the disease to start getting worth. The average survival time in GBM Patients was 14.6 months when they received Temodal and radiotherapy, compared with 12.1 months with radiotherapy alone.

The biological activity of TEMODAR in pediatric patients has not been identified. Phase II studies on TEMODAR Capsules have been conducted in pediatric patients (age 3-18 years) at a dose of 160-200 mg/m 2 times daily for 5 days every 28

days. A clinical trial was conducted by the Schering Corporation in which 29 patients with recurrent brain stem glioma and 34 patients with recurrent high grade astrocytoma were involved. Another Phase 2 open label study was conducted by the Children's Oncology Group (COG) in which 122 patients were enrolled. The clinical trials showed the same toxicity profile for TEMODAR in pediatric and adult patients (FDA TEMODAR® (temozolomide) capsules, 2006).

Resistance to temozolomide is mediated by the enzyme O6-alkylguanine transferase AGT(Stupp, Gander, Leyvraz, & Newlands, 2001). GBM Patients who have inactivated AGT (methylated promoter) achieve more clinical benefits from TMZ than patients with activated AGT (non-methylated promoter) (Esteller et al., 2000).

Standard carcinogenicity and reproductive function studies were not conducted with temozolomide. Mammary carcinomas were observed in both male and female rats after they were treated with 200 mg/m^2 temozolomide on 5 days every 28 days for 3 cycles. Mammary carcinomas and fibrosarcomas of different body organs were observed after 6 cycles of receiving temozolomide at 25, 50, and 125 mg/m^2. In vitro mutagenicity was observed in bacteria (Ames assay). Clastogenicity in mammalian cells was observed (human peripheral blood lymphocyte assays). Multicycle toxicology studies in rats and dogs have indicated testicular toxicity (syncytial cells/immature sperm, testicular atrophy) at doses of 50 mg/m^2 in rats and 125 mg/m^2 in dogs (FDA TEMODAR® (temozolomide) capsules, 2006).

Temozolomide received an accelerated approval by the U.S. Food and Drug Administration in January 1999 for the treatment of anaplastic astrocytoma patients who experienced a resistance for nitrosourea and procarbazine (W. A. Yung et al., 1999). The

drug received full approval after the clinical benefits for temozolomide and radiotherapy were compared with radiotherapy alone. On March 15, 2005, the FDA approved temozolomide combined with radiotherapy for the treatment of Glioblastoma Multiforme (Cohen, Johnson, & Pazdur, 2005). The European Committee for Medicinal Products for Human CHMP decided that Temodal's benefits are greater than its risks. Temodal received approval for the treatment of Glioblastoma Multiforme from The (CHMP) on 26 January 1999.

2.9 Immune Reactions in Healthy Brain

The Blood Brain Barrier BBB is a unique, selective barrier which consists of the endothelial cells and perivascular elements such as closely associated astrocytic end-feet, perivascular neurons and pericytes as shown in figure 2.4 (a). Several membrane transporters are present in the brain endothelial cells. They are responsible for the regulation of the penetration of essential molecules from the blood circulation into the brain. Membrane transporters are also responsible for the effluxing of potentially harmful substances and waste products out of the brain cells. P-glycoprotein (P-gp) and the multidrug resistance-associated protein family are the most important efflux transport systems responsible for drug delivery to the CNS. The presence of complex tight junctions (TJ) and adherens junctions (AJ) makes the cerebral endothelial cells very unique. TJs prevent the penetration of polar molecules into the brain, however AJs stabilize cell–cell interactions in the junctional zone. In addition, there are intracellular and extracellular enzymes such as monoamine oxidase (MAO), γ-glutamyl transpeptidase (γ-GT), alkaline phosphatase, peptidases, nucleotidases and several cytochrome P450

enzymes which provide the BBB with the metabolic activity as shown in figure 2.4 (b) (Cecchelli et al., 2007).

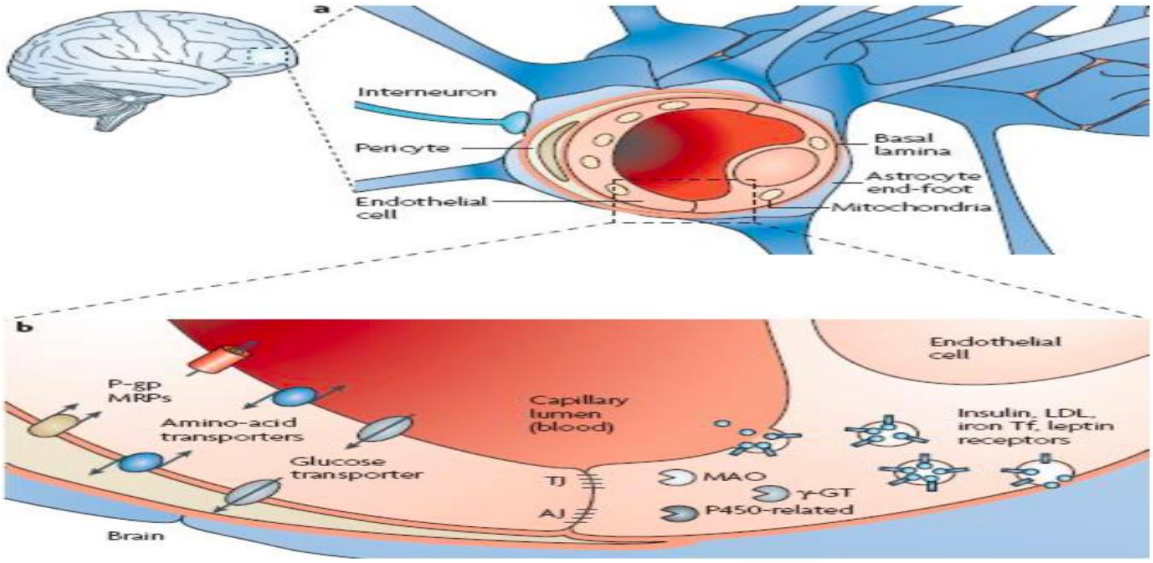

Figure 2.4 The cells of the BBB (a) and the existing membrane transporters (b) (Cecchelli et al., 2007).

It was thought that immune reactions do not occur in the brain due to the specific nature of the blood brain barrier. The brain has specific characteristics such as the absence of conventional lymphatic vessels and decreased number of circulating T cells. It is recently known that the central nervous system communicates with the immune system by the means of two-way communication pathways. Recent models such as infectious or experimental autoimmune encephalomyelitis animal models provide information about the specific operations of the immune system in the brain (Vauleon, Avril, Collet, Mosser, & Quillien, 2010).

A healthy brain contains several different immune cell populations. Microglial cells resemble 5% to 20% of cells in the central nervous system starting from the embryonic development. Microglial cells arise from hematopoietic cells and are considered as the first line of defense in the brain. Macrophages and dendritic cells (DC) are present in perivascular zones, the choroid plexuses, and the meninges. Macrophages and dendritic cells arise from the monocytes which are circulating in the blood stream (Vauleon et al., 2010).

Microglial cells travel to the inflammatory zones. undergo activation, obtain phagocytic properties, and produce different types of cytokines and chemokines which synthesise other types of immune cells (Tambuyzer, Ponsaerts, & Nouwen, 2009). The T cells are being activated in the cervical nodes, resulting in increasing the levels of $\alpha 4/\beta 7$ integrins. Animal models showed that antigen presenting cells (APC) travel from the brain parenchyma through the external capsule to enter the cervical nodes which is the same drainage system pathway in humans and rodents (Karman, Ling, Sandor, & Fabry, 2004).

The blood brain barrier exhibits a selective penetration of immune cells from the blood circulation into the brain parenchyma due to the complex cellular structure of the brain capillaries such as; endothelial cells with tight junctions, pericytic cells, and astrocytic cells. The penetration of the activated T cells across the blood brain barrier is controlled by rolling, activation, adhesion, and transmigration.

The process is also controlled by several molecular interactions involving adhesion molecules such as α4/β7 integrins and chemokines such as CXCR3 (Mrass & Weninger, 2006; Wilson, Weninger, & Hunter, 2010).

2.10 Immune Reactions in GBM patients

It is obvious that the rules that regulate the penetration of the effector T cells across the tumour tissues and the normal state rule are different (Vauleon et al., 2010). The blood brain barrier in GBM patients has abnormal asymmetric capillary structure. The tight junctions between endothelial cells are inactivated and the blood brain barrier associated pericytes are decreased in number (Mrass & Weninger, 2006; Davies, 2002; Rascher et al., 2002). The travelling of lymphocytes was tracked with injections of CD4+ lymphocytes in a model of autoimmune encephalitis to target myelin proteins.

$CD4^+$ lymphocytes entered the subarachnoid spaces and travelled through the internal wall first then through the external walls of the vessels. When $CD4^+$ lymphocytes identify any Antigen Presenting Cells APC such as macrophages or dendritic cells in the myelin antigens, they become reactivated, produce numerous cytokines, and enter the brain parenchyma (Bartholomäus et al., 2009). $CD8^+$ lymphocytes are locally expanded depending on the brain environment. Glioma mouse model showed that $CD8^+$ lymphocytes first enter the brain, then undergo proliferation and differentiation depending on IFNγ and granzyme B expression (Vauleon et al., 2010). $CD8^+$ T cells have the ability to retain into the brain due to the presence of $\alpha E\beta 7$ integrins (Masson et al., 2007). This increased level of $\alpha E\beta 7$ integrins is thought to be resulting from the presence of TGFβ (Vauleon et al., 2010). The infection models showed that $CD8^+$ T cells also have

the ability to expand in the brain depending on the overexpression of the dendritic cells (Lauterbach, Zuniga, Truong, Oldstone, & McGavern, 2006).

2.11 Immunotherapy for Glioblastoma Multiforme

The occurrence of effective anticancer immune response is initiated and maintained by a series of stepwise events called the cancer-immunity cycle as shown in figure 2.5. In step 1, neoantigens are formed and released by oncogenesis and captured and processed by dendritic cells (DCs). In step 2, DCs present the captured antigens on MHCI and MHCII molecules to T cells. T cell responses against the cancer-specific antigens are primed and activated in step 3. In steps 4, 5, 6, and 7, the activated effector T cells traffic to, infiltrate the tumor bed, specifically recognize, bind to, and kill their target cancer cell. The cancer-immunity cycle is iterative because the death of the cancer cell releases additional tumor-associated antigens which strengthen the previously explained immune response (D. S. Chen & Mellman, 2013). The Cancer-Immunity Cycle is limited in GBM patients because the tumor antigens may not be detected and DCs and T cells may not recognize antigens as foreign. Moreover, T cells may not properly home to tumors, or may be inhibited from infiltrating the tumor. The suppression of those effector cells by tumor microenvironment is another important factor that also may limit the Cancer-Immunity Cycle (Motz & Coukos, 2013).

The presence of checkpoints and inhibitors in each step of the cancer-immunity cycle may negatively affect the production of the antitumor immune response. Checkpoints of cancer-immunity cycle are common rate-limiting steps and exhibit

immunostat function that takes place in the tumor microenvironment and cause immunosuppression (Jarrod Predina et al., n.d. ; Wang et al., n.d.). Each step of the Cancer-Immunity Cycle has stimulatory; which initiate and maintain immunity and inhibitory factors; which inhibit immunity and also prevent autoimmunity. For example, CTLA4 is an immune checkpoint protein; which works on step 3 to prevent the development and proliferation of t cells and hinder the desired active immune response. There are also immune rheostat (immunostat) factors, such as PD-L1; which acts on step 7 and modulate active immune responses in the tumor bed. Stimulatory and inhibitory factors of Cancer-Immunity Cycle are shown in table 2.1 .These factors include " IL, interleukin; TNF, tumor necrosis factor; IFN, interferon; CDN, cyclic dinucleotide; ATP, adenosine triphosphate; HMGB1, high-mobility group protein B1; TLR, Toll-like receptor; HVEM, herpes virus entry mediator; GITR, glucocorticoid-induced TNFR family-related gene; CTLA4, cytotoxic T-lymphocyte antigen-4; PD-L1, programmed death-ligand 1; CXCL/CCL, chemokine motif ligands; LFA1, lymphocyte function-associated antigen-1; ICAM1, intracellular adhesionmolecule1; VEGF, vascular endothelial growth factor ;IDO,indoleamine2,3-dioxygenase; TGF, transforming growth factor; BTLA,B-and T-lymphocyte attenuator; VISTA, V-domain Ig suppressor of T cell activation; LAG-3, lymphocyte-activation gene 3 protein; MIC, MHC class I polypeptide-related sequence protein; TIM-3, T cell immunoglobulin domain and mucin domain-3". These factors arise from the intratumoral T regulatory cells, macrophages, and myeloid-derived suppressor cells. There are also specific immunogenic signals such as proinflammatory cytokines and factors released by dying tumor cells that are crucial for the production of an anticancer T cell response (D. S. Chen & Mellman, 2013).

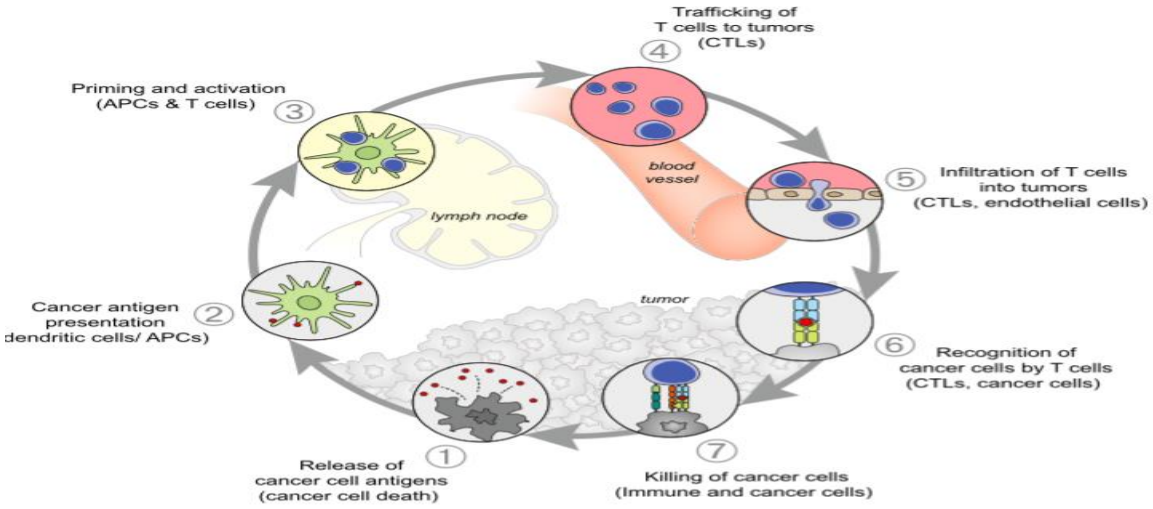

Figure 2.5 The cancer-immunity cycle (D. S. Chen & Mellman, 2013).

Table 2.1 Positive and negative regulators of the cancer-immunity cycle (D. S. Chen & Mellman, 2013).

Steps	(+) Stimulators	(–) Inhibitors	Other Considerations	Example References
1. Release of cancer antigens	Immunogenic or necrotic cell death	Tolergenic or apoptotic cell death	Tumor-associated neoantigens and cancer testis antigens	Ferguson et al., 2011
2. Cancer antigen presentation	• Proinflammatory cytokines (e.g., TNF-α, IL1, IFN-α) • Immune cell factors: CD40L/CD40 • Endogenous adjuvants released from dying tumors: CDN (STING ligand), ATP, HMGB1 • Gut microbiome products: TLR ligands	IL-10, IL-4, IL-13	Dendritic cell maturity	Lippitz, 2013; Mellman et al., 2011
3. Priming and activation	CD28:B7.1, CD137 (4-1BB)/ CD137L, OX40:OX40L, CD27:CD70, HVEM, GITR, IL-2, IL-12	CTLA4:B7.1, PD-L1:PD-1, PD-L1:B7.1, prostaglandins	Central tolerance, T cell repertoire, T regulatory cells	Franciszkiewicz et al., 2012; Lippitz, 2013; Riella et al., 2012; So et al., 2006
4. Trafficking of T cells to tumors	CX3CL1, CXCL9, CXCL10, CCL5			Franciszkiewicz et al., 2012; Peng et al., 2012
5. Infiltration of T cells into tumors	LFA1:ICAM1, selectins	VEGF, endothelin B receptor		Franciszkiewicz et al., 2013
6. Recognition of cancer cells by T cells	T cell receptor	Reduced peptide-MHC expression on cancer cells		Mellman et al., 2011
7. Killing of cancer cells	IFN-γ, T cell granule content	PD-L1:PD-1, PD-L1:B7.1, TIM-3:phospholipids, BTLA, VISTA, LAG-3, IDO, Arginase, MICA:MICB, B7-H4, TGFβ	T regulatory cells, myeloid-derived suppressor cells, M2 macrophages, hypoxia	Chen et al., 2012; Greaves and Gribben, 2013; Mellman et al., 2011; Topalian et al., 2012a

The use of immunotherapy for the treatment of brain tumors consists of two options. The first option is the use of active immunotherapy that aims to increase the native immune response in Glioblastoma patients. The second option is the use of passive immunotherapy to target the tumor cells. Passive immunotherapy consists of in vitro activated immune cells or specific molecules such as antibodies which can be injected directly (Vauleon et al., 2010).

2.12 Adoptive Immunotherapy

The in vitro activated immune cells are either being directly injected into the tumor tissues or intravenously in GBM patients (Vauleon et al., 2010). Lymphocyte-activated killer cells are used for the treatment of Glioblastoma patients (Jacobs, Wilson, Kornblith, & Grimm, 1986; Robert Owen Dillman et al., 2009). There are several clinical trials that were conducted on a number of High Grade Glioma patients and based on the use of lymphocyte activated killer cells LAK (Vauleon et al., 2010). The toxic effects of LAK include neurological toxicity and brain edema (Barba, Saris, Holder, Rosenberg, & Oldfield, 1989). Radiological response criteria were used to evaluate the improvement after the treatment was received by patients. One hundred and eighteen GG patients were enrolled in some LAK cells clinical trials; five patients were reported with a complete response, 13 patients with a partial response, and six patients with stable disease (Vauleon et al., 2010). Vaccinated Glioblastoma patients achieved a longer median survival than the median survival of the control groups (Hayes et al., 1995; Hayes et al., 2001; Robert O. Dillman et al., 2004). In one clinical trial, the median overall

survival of a newly diagnosed GBM patients reached 20.5 months and one year for 75% of the patients (Robert Owen Dillman et al., 2009).

Cytotoxic T lymphocytes (CTL) are also used for the treatment of GBM patients (Vauleon et al., 2010). Several clinical trials were conducted to evaluate the efficacy of cytotoxic T lymphocytes (CTL) on High Grade Glioma patients. Cytotoxic T Lymphocytes were delivered to the cerebrum by direct injection into the tumor site (Vauleon et al., 2010). In some clinical trials, cytotoxic T lymphocytes were generated from lymph nodes or peripheral blood mononuclear cells and were combined with vaccination strategy in 65 patients. The combination strategy of both immune cells were delivered to the tumor site either by intravenous injection or by intracarotid infusion. The reported side effects include a hypersensitivity reaction at the injection site. The degree of tolerance was acceptable and no disease progression was reported (Vauleon et al., 2010). Ninety-five patients were treated with this combination therapy and were assessed as follows: 18 patients with a stable disease state, 28 patients with partial response, and three patients with complete response. One clinical trial has reported a survival time up to eight months in seven of 15 patients and up to 40 months in one patient (Holladay, Heitz-Turner, Bayer, & Wood, 1996).

2.13 Active Immunotherapy

There are different cells such as intact tumor cells, tumor protein lysates, tumor-derived mRNA, and natural and synthetic peptides which can be used as antigen sources for the active immunotherapy. Antigens can be used alone or with dendritic cells and combined with other adjuvants to increase the immune response (Vauleon et al., 2010).

Autologous tumor cells (ATC) was used as vaccines in several clinical trial studies for the treatment of High Grade Glioma patients. The cells are injected either subcutaneously or intradermally. Several clinical studies reported that the vaccination procedure should be repeated for three cycles with a total of one to 13 injections. Side effects include fever, erythema, and abnormal liver tests (Vauleon et al., 2010). Fifty-three high Grade Glioma patients were enrolled in several clinical studies based on the treatment with autologous tumor cells and the patients were assessed as follows: four patients with complete response, six patients with partial response, two patients with minor response, and six patients with stable disease.

Vaccination based on dendritic cells is very useful for the treatment of GBM especially for patients with small tumors. The antigens were derived from tumor lysates, peptides or mRNA that are obtained from autologous tumor cells ATC or the whole ATC in several clinical studies. Tolerance was manageable with the rare incidence of grade four neurotoxicity (Yamanaka et al., 2005). Side effects include; headache, seizure, and flu-like syndrome. This treatment strategy was found to initiate a peripheral immune response in large percent of patients who were enrolled in the clinical studies.

Further investigation on a number of patients who received vaccination showed that CD8 lymphocytes were specifically expressed. The analysis of the radiological response of c Thirty-four Glioblastoma patients who have responded to the vaccination treatment showed a major increase in IFNγ production, however no overproduction was detected in nonresponsive patients. The responded patients also experienced an enhanced response to the chemotherapy which was administered in a second phase (Wheeler et al., 2008). When dendritic cells were exposed to the proper antigens, they enabled the

adaptive immune system to produce immune response and eradicate tumor cells (Wang et al., 2014).

Early vaccinated Glioblastoma patients with high intratumoral infiltration by T lymphocytes were found to experience a small level of intratumor TGFβ and longer survival. Patients with major tumors who were vaccinated late showed no infiltration in T lymphocytes, experienced high levels of intratumor TGFβ, and achieved less than 12 months of overall survival (Liau et al., 2005). Glioblastoma patients who have received mature dendritic cells achieved longer survival than patients who have received immature dendritic cells. The co- administration of both peripheral and intracranial dendritic cells showed a more optimum response than peripheral injection only (Yamanaka et al., 2005).

A combined therapy of dendritic cells (DC) that was obtained from monocytes and temozolomide in a primary treatment course of therapy was tested on 14 Glioblastoma patients. Two patients were reported with positive antitumor immune responses in the peripheral blood (Hunn et al., 2015).

2.14 DC Development, Diversification, Maturation, and Function

Dendritic cells (DCs) are antigen presenting cells, which have the ability to stimulate the native T cells and induce primary immune responses and peripheral immunological tolerance. Immature DCs (im DCs) originates from hematopoietic stem cells within the bone marrow. The process of development and diversification of DCs is antigen-independent. $CD34^+$ hematopoietic stem cells differentiate into common myeloid progenitor cells (CMP) and common lymphoid progenitor cells (CLP). The CMPs differentiate into $CD34^+$ CLA^+ and $CD34^+$ CLA^- late progenitor cells. While $CD34^+$

CLA^+ cells differentiate into $CD11c^+$ $CD1a^+$ Langerhans cell precursors, $CD34^+CLA^-$ cells differentiate into $CD11c^+$ $CD1a^-$ interstitial DC precursors in blood. The blood $CD11c^+$ $CD1a^+$ Langerhans cell precursors migrate into the skin epidermis and become Langerhans cells and the $CD11c^+CD1a^-$ migrate into the skin dermis and other tissues to become interstitial DCs. In case of the absence of antigen/pathogen stimulation, both Langerhans cells and interstitial DCs play a critical role in immune tolerance in the lymph nodes. After being stimulated by microbes, Langerhans cells and interstitial DCs become mature and rapidly induce primary immune responses. CMP and CLP also differentiate into Phenotype myeloid pre-DC1s and lymphoid pre-DC2s in bone marrow. After bacterial infection, pre-DC1s recognize and destroy bacteria, differentiate into DCs, and initiate adaptive antibacterial immune responses. After viral infection, pre-DC2s triggers the production of type-1 IFN, differentiate into DCs, and initiate adaptive antiviral immune responses as shown in figure 2.6 (Yong-Jun Liu, n.d.).

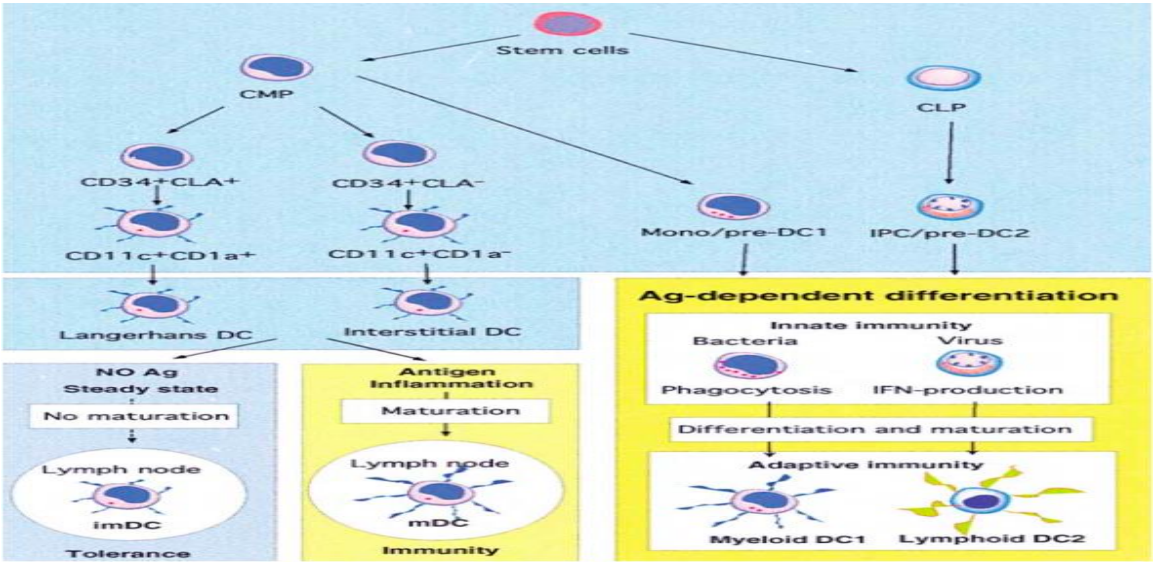

Figure 2.6 DC Development, Diversification, Maturation, and Function (Yong-Jun Liu, n.d.).

2.15 Tumor Associated Antigens TAA and Tumor Targets

There are many Tumor Associated Antigens TAA or tumor antigen precursor proteins TAPP which were found to be expressed on different GBM cell lines as shown in table 2.2 (Zhang et al., 2007) (Saikali et al., 2006). TAAs or TAPP are also overexpressed on the Cancer stem cells CSC (Xu et al., 2009). TAA are known to stimulate human immune responses and can be used to make autologous dendritic cell vaccines (Zhang et al., 2007).

Table 2.2 TAA expression profiles on GBM cell lines (Zhang et al., 2007).

(A) Intracellular flow cytometric analysis of 12 TAPP expressed within the glioma cells*

Antigen	Glioma											
	A172	D-54	LN18	LN229	LNZ308	SF767	SNB19	T98G	U-118	U-251	U-373	U-87
B-cyclin	1	1	2	2	2	2	3	1	1	1	2	1
EphA2	1	1	1	2	2	1	1	0	1	2	3	1
Her2/neu	2	3	3	3	3	2	3	2	2	3	3	3
IL13Rα2	3	4	2	2	3	2	3	2	3	3	3	3
GnT-V	2	1	2	2	1	2	2	3	1	3	2	2
GP100	3	2	0	0	3	2	3	3	0	3	4	3
Mage-1	4	3	2	3	3	3	3	2	2	3	2	3
Mart-1	3	0	3	3	3	2	3	3	0	3	3	3
Survivin	3	2	2	2	3	2	2	3	1	3	2	3
hTert	4	4	3	3	4	4	2	4	4	4	3	4
Trp-1	0	2	1	1	0	0	1	1	0	0	1	0
Tyrosinase	2	0	2	3	1	2	2	3	0	2	0	0
Σ score	28	23	23	26	28	24	28	27	15	30	28	26

(B) Real-time PCR expression of Aim-2, Gage-1, Sart-1, and Trp-2 mRNA†

Antigen	Glioma											
	A172	D-54	LN18	LN229	LNZ308	SF767	SNB19	T98G	U-118	U-251	U-373	U-87
Aim-2	2	3	3	3	3	2	3	3	3	3	3	2
Gage-1	nd	nd	nd	nd	nd	nd	nd	nd	2	nd	nd	nd
Sart-1	2	2	3	2	nd	1	2	2	2	1	2	nd
Trp-2	nd	nd	nd	2	nd	2	nd	2	2	nd	1	nd
Σ score	4	5	6	7	3	5	5	7	9	4	6	2
Total‡	32	28	29	33	31	29	33	34	24	34	34	28

The defined TAAs also include HER2/neu (Zhang et al., 2007; Liu et al., 2004), TRP-2 (Zhang et al., 2007; Saikali et al., 2006; "Molecular and Functional Analysis of

Tyrosinase-Related Prot...," n.d.), AIM-2 (Zhang et al., 2007; "AIM-2," n.d.), gp100, MAGE1, and Interleukin 13 receptor α2-chain IL13Ra2 (Zhang et al., 2007; Liu et al., 2004; Okano, Storkus, Chambers, Pollack, & Okada, 2002).

Furthermore, CD133 is a stem cell marker which is overexpressed on GBM cells and was found to result in radio- resistance, chemo-resistance, and tumor aggressiveness. CD133 was found to be increased by 4.6-fold in recurrent GBM cells compared with the percentage in primary GBM cells (Pallini et al., 2011). Several studies have shown that cytomegalovirus phosphoprotein 65pp65 HCMV is also expressed in more than 90% of GBM specimens in contrast with normal brain (Dziurzynski et al., 2012; Cobbs et al., 2002); which suggests that HCMV viral proteins may be used as tumor-specific target (Mitchell et al., 2015).

2.16 End Points and Evidences of Therapeutic Activity

One of the primary end points is the Overall survival (OS) which was defined as "the *time from the day of surgical tumor resection until the date of death due to any cause*". Another end point is the Progression-free survival (PFS) which was defined as "*the time from the day of surgical tumor resection until the first documented progression in MRI or death due to any cause whichever is earlier*" (Akasaki et al., 2016).

Objective tumor response is considered as a targeted end point in some clinical trials. It is measured by investigating the presence of a complete response, partial response, progressive disease, or stable disease. Complete response (CR) is defined as the complete disappearance of all lesions for 4 weeks. Partial response (PR) is defined as the

50 % reduction in tumor size for 4 weeks. Progressive disease (PD) is defined as 25 % increase in tumor size or appearance of new lesions. Stable disease (SD) is neither PD or PR as shown in 2D categorization criteria shown in table 2.3 (Miller, Hoogstraten, Staquet, & Winkler, 1981).

Table 2.3 1D and 2D response categorization criteria (Shah et al., 2006).

Method	**Response Category**			
	% CR	% PR	% SD	% PD
1D	100	>30	30 to 20	>20
2D	100	>50	25 to 25	>25

2.17 Nanotechnology in Immunotherapy for GBM

There is a great need for an optimized therapy for the treatment of GBM. Although, immunotherapy is considered a potential treatment for glioblastoma, it still faced by major limitations such as target identification, delivery system, and local suppression of the immune system (Patel, Kim, Ruzevick, Li, & Lim, 2014). The blood brain barrier blood brain barrier BBB prevents large particles from penetrating into the brain to be delivered to the tumor site. Increasing the dose of the therapeutics will cause toxic side effects due to the decreased local bioavailability and the accumulation of the medicines into the vital organs through the reticuloendothelial system (Ung & Yang, 2015). Nanotechnology can be used to increase the efficacy of new therapeutics and decrease the toxic side effects (Nduom, Bouras, Kaluzova, & Hadjipanayis, 2012). The particle size, morphology, composition, and surface modifications of the nanoparticles can be controlled and modulated according to the intended use and the selected target as

shown in Figure 2.3. Nanoparticles are synthesized from carbon, lipids, or polymeric units can carry and deliver different active ingredients locally to the tumor cells and avoid normal cells. Nanoparticles can enhance the efficacy of the immunotherapy that is used as a potential treatment for glioblastoma Multiforme. Nanoparticles can selectively target interleukin-13 receptors which are overexpressed in the tumor cells. This mechanism of selective targeting has a great advantage of minimizing toxic side effects especially in the healthy tissues (Ung & Yang, 2015).

Dendritic nanoparticles or dendrimers consist of large number of oligomeric branches and several surface groups. They can be modified due to the large number of branching units using a wide range of functional groups to improve the immunotherapy for Glioblastoma Multiforme. Dendrimers were grafted to both doxorubicin and siRNA (Ofek, Fischer, Calderón, Haag, & Satchi-Fainaro, 2010). Dendritic nanoparticles achieved high degree of selectivity and no cytotoxicity effects were reported. The use of siRNA loaded in a nanoparticle is considered a potential treatment especially in the gene therapy approach for the treatment of GBM (Ofek, Fischer, Calderón, Haag, & Satchi-Fainaro, 2010). Nanoparticles is a potential solution which can be used to overcome the major limitations of the immunotherapy for GBM. They exhibit a great ability to attach efficiently with the targeted sites, deliver different treatments in a concentrated and controllable manner to the tumor tissues, and reducing the toxic side effects (Ung & Yang, 2015).

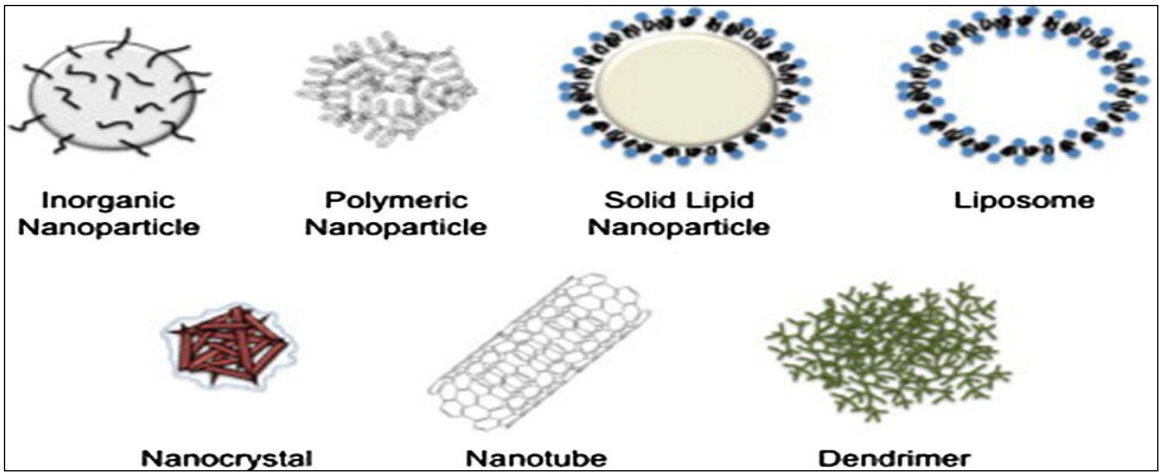

Figure 2.7 Different shapes and compositions of nanoparticles that are used in drug delivery (Faraji & Wipf, 2009).

2.18 Summary

This chapter has provided an overview to the review of relevant literature. It has provided Glioblastoma Multiforme (GBM), epidemiology, metastases, cancer stem cell hypothesis, treatment options "Radiotherapy, Bevacizumab, and Temozolomide", immune reactions in healthy brain and in Glioblastoma patients, immunotherapy for the treatment of GBM, adoptive immunotherapy, active immunotherapy, DC development, diversification, maturation, and function and nanotechnology in immunotherapy for the treatment of GBM. It also has introduced an overview of the tumor associated antigens TAAs, and end points and evidences of therapeutic activity.

CHAPTER 3. METHODOLOGY

This chapter provides the methodology of investigating the DC- based vaccination immunotherapy option for GBM that includes data collection procedures, data sources, and data analysis techniques.

3.1 Data Collection

This study is conducted to explore the clinical outcomes of DC vaccines; which are currently under investigation for the treatment of GBM. Clinical trials showed that treating GBM patients with surgery, TMZ, and Dendritic Cell DC vaccines was safe and achieved meaningful clinical outcomes. The sources of antigen that have been used in DC immunotherapy include exogenous MHC-restricted peptides, acid-eluted tumor peptides, tumor cell lysate, and whole glioma cells. The study is exploring the efficiency of the treatment combination of immunotherapy, chemotherapy, following surgical resection of the tumor mass based on OS and PFS values. Data about the inclusion criteria of GBM in published clinical trials, pre and post vaccination treatment strategies, antigen sources, and immune responses that are related to the relatively most preferable clinical outcomes (OS and PFS) has been considered.

3.2 Data Sources

Data will be collected from:

- ✓ The official site of the Food and Drug Administration Agency.
- ✓ The official site of the European Medicines Agency.
- ✓ Clinical trials websites.
- ✓ The published literature of the clinical trials; which were conducted on GBM patients.

3.3 Data Analysis

An independent samples t-test has been used to compare the means of OS and PFS values of independent sample groups of GBM patients. Data has been collected from published results of the clinical trials conducted on GBM patients who were involved in different treatment strategies. Moreover, ANOVA test has been performed to compare the means of OS and PFS values of two or more independent sample groups of GBM patients and to show graphical displays.

The first independent samples t-test has been performed to determine the presence of a statistically significant difference of OS and PFS values between 2 groups of patients. The first group received DC based vaccination pulsed with specific antigens (SA) with concomitant or post vaccination TMZ. The sources of SA antigens include the synthetic class I peptides AIM-2, MAGE1, TRP-2, gp100, HER2/neu, and IL-13Ra2 or the synthetic peptides TRP-2, gp100, her-2/neu, and survivin. The second group received DC based vaccination loaded with tumor material antigens (TM) and concomitant or post vaccination TMZ. The sources of TM antigens include autologous tumor or glioma cells.

The second independent samples t-test has been performed to determine the presence of a statistically significant difference of OS and PFS values between two groups of GBM patients. The first group have received DC vaccine + concomitant or post

vaccination TMZ and did not receive pre-vaccination TMZ (Nil). The second group have received pre-vaccination TMZ + DC vaccine + concomitant or post-vaccination TMZ (TMZ).

The third independent samples t-test has been performed to determine the presence of a statistically significant difference of OS values between two groups of GBM patients. The first group received pre-vaccination TMZ + RT + DC vaccine + concomitant or post-vaccination TMZ (TMZ+RT). The second group of GBM patients DC vaccine + concomitant or post vaccination TMZ and did not receive pre-vaccination therapy (Nil).

The fourth independent samples t-test has been performed to determine the presence of a statistically significant difference of PFS values between two groups of GBM patients. The first group received pre-vaccination TMZ + RT + DC vaccine + concomitant or post-vaccination TMZ (TMZ+RT). The second group of GBM patients received DC vaccine + concomitant or post vaccination TMZ and did not receive pre-vaccination therapy (Nil).

ANOVA test has been performed to determine the presence of a statistically significant difference between the means of OS and PFS values of two groups of GBM patients. The first group includes patients who received DC based vaccination + concomitant or post-vaccination TMZ with or without RT. The second group includes patients who received TMZ+RT with no DC vaccine.

3.4 Summary

This chapter has presented the different elements of the research methodology. It shows data collection procedures, data sources, and data analysis techniques of this research.

CHAPTER 4. DATA COLLECTION, DATA ANALYSIS, AND FINDINGS

This chapter presents the data which has been collected to investigate the clinical outcomes of using different DC- based vaccination strategies, immune responses that are accompanied by the most preferable clinical outcomes, data analysis, and findings.

4.1 Data Collection

Patients' information, the inclusion criteria in published clinical trials, pre and post vaccination treatment strategies, antigen sources, immune responses that are related to the relatively most preferable clinical outcomes (OS and PFS) have been collected.

4.1.1 Clinical Outcomes

Different clinical outcomes were associated with DC- based vaccination therapy loaded with different antigens which were obtained from different antigen sources. The sources of antigen that have been used in DC immunotherapy include synthetic class I peptides AIM-2, MAGE1, TRP-2, gp100, HER2/neu, and IL-13Ra2, synthetic peptides TRP-2, gp100, her-2/neu, and surviving, and autologous tumor or glioma cells. Clinical trials showed that treating GBM patients with surgery, TMZ, and Dendritic Cell DC vaccines was safe and achieved meaningful clinical outcomes. OS and PFS are the most

widely used end point in clinical trials. Different values of OS and PFS reached by GBM patients involved in different treatment strategies have been investigated.

4.1.1.1 Conventional Treatment with TMZ+RT

Data were obtained from the (EORTC 26981/22981) phase 3 clinical trial; which was conducted to prove the efficacy and safety of TMZ combined with RT in comparison to RT alone. This trial included 573 patients, 287 were treated with RT+TMZ and 286 patients were treated with RT alone. Kaplan Meier curves show the survival distributions achieved with RT + TMZ. The median overall survival was 14.6 months and the median PFS was 6.9 months for patients who were treated with TMZ+RT. 61% of GBM patients reached 1-year OS and 26% achieved 2 years OS after being treated with RT + TMZ. The efficacy results are shown in figure 4.1 and figure 4.2 (EMA, n.d.).

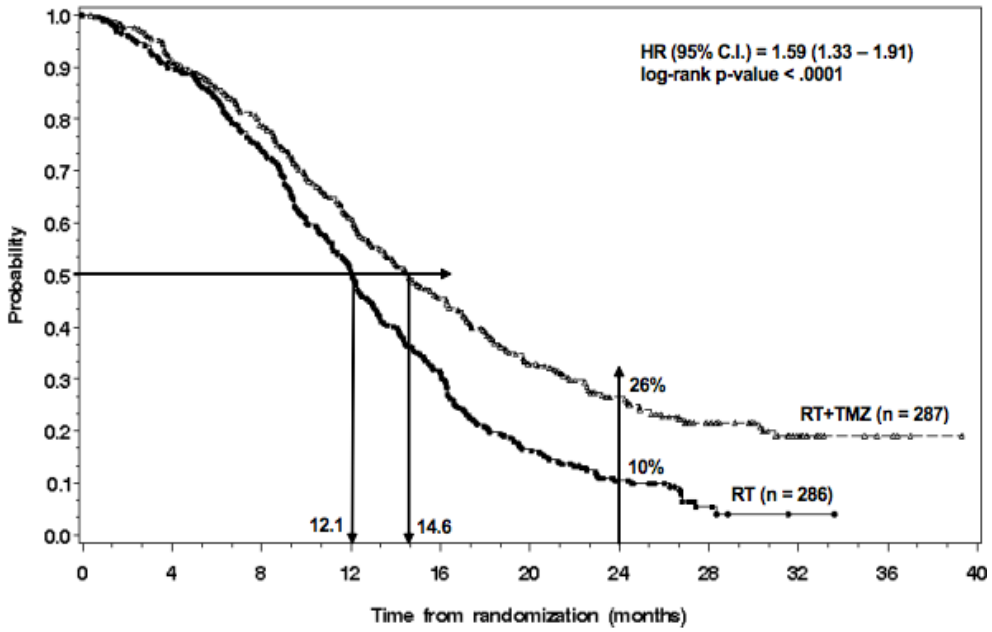

Figure 4.1 Kaplan-Meier Curves for Overall Survival with (TMZ+RT) (EMA, n.d.).

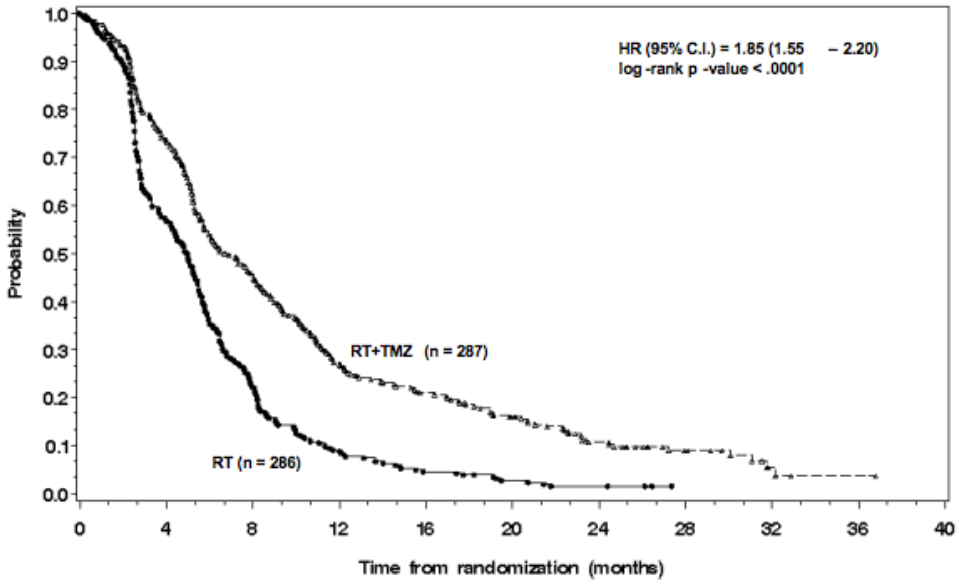

Figure 4.2 Kaplan-Meier Estimates for Progression-Free Survival with TMZ+RT (EMA , n.d.).

4.1.1.2 DC Vaccine improves Responses to Chemotherapy

The treatment of GBM patients with DC vaccine in combination with standard adjuvant therapy has showed safety and efficacy. The values of overall survival of 13 patients with malignant astrocytoma who were treated with DC vaccination and conventional therapy are shown in table 4.1. The age of recruited patients varied between 1–75, the performance status ECOG (Eastern Cooperative Oncology Group) was 0, 1 or 2 (Walker et al., 2008). The ECOG performance status of 0, 1, and 2 is equivalent to ≥ 70% of Karnofsky performance status (Oken et al. 1982). Three out of eight GBM patients had progressive disease and five patients showed an objective radiological response to treatment. One patient showed a complete response, which persisted for three months. There were no adverse events attributed to the use of DC vaccines. This study

demonstrated that the improvements of patient responses to adjuvant chemotherapy were related to the use of DC vaccines (Walker et al., 2008).

4.1.1.3 Clinical Outcomes of the Treatment Strategy of DC Vaccine loaded with Tumor Material (TM) and Chemotherapy

A phase 1 clinical study showed that treating GBM patients with surgery, TMZ, autologous tumor material loaded DC vaccines, and further TMZ was safe and achieved a limited efficacy as shown in table 4.2. Information regarding enrolled patients and previous treatment strategies are shown in Table 4.3. The 6 months PFS for all treated patients (n =9) was 22 % which is similar in the combined treatment and the treatment with TMZ alone. The median OS from the original diagnosis of GBM for the treated patients was 23 months. No grade IV toxicities or DC related toxicity were detected. The reported adverse events of the combined therapy were only attributed to the anticipated toxicity of TMZ (Hunn et al., 2014).

A clinical trial was designed to evaluate the safety and clinical responses of two groups of GBM patients treated with a combined therapy of TMZ and fusions of DCs and glioma cells (FC). Group-R included patients (n = 10) with recurrent GBM patients after failing TMZ-chemotherapy and Group-N included newly diagnosed GBM patients (n = 22). The selection criteria included a Karnofsky performance status (KPS) of $\geq$ 50 % and the age average was 54.6 years.The medians, first quartiles, third quartiles, and 95 % CIlow of medians were evaluated as shown in figure 4.3 (Akasaki et al., 2016).

Table 4.1The results of a phase I dendritic cell vaccine and standard adjuvant therapy trial for malignant astrocytoma (Walker et al., 2008).

Patient code (age/sex)	Date of surgical resection(s)	Histology	Number of vaccines	Pre-vaccination treatment	Post-vaccination treatment	Response to chemotherapy	Survival post-enrolment
DG03 (66F)	Jul03 Sep03	GBM	6	Nil	TMZ (2 cycles)	Partial	12m
DG04 (71M)	Aug03	GBM	2	Nil	Palliative XRT	N/A	2m
DG05 (25F)	Sep03	GBM	6	Resection LGA,Sep03	Nil	N/A	4m
DG06 (52F)	Oct03	GBM (rec)	4	Resection GBM, May02	Nil	N/A	5m
DG07 (44M)	Oct03 Aug04	GBM	13	Resection LGA, Nov92 with post-resection XRT	TMZ (6 cycles)	Nil	18m
DG08 (66M)	Jun04	GBM	6	Nil	XRT	N/A	9m
DG09 (47M)	Jun04	GBM	10	Nil	TMZ	Partial	15m
DG10 (60M)	Sep04 May05	GBM	9	XRT/TMZ	TMZ	Nil	11m
DG11 (55M)	Nov04	GBM (rec)	5	Resection GBM, Apr04 with post-operative XRT/chemo	TMZ (2 cycles)	Nil	5m
DG12 (51F)	Jan05	AA (rec)	9	Resection AA, Oct01 with post-operative XRT and TMZ	TMZ (6 cycles)	Complete	25m
DG13 (36M)	Feb05 Jul05	AA (rec)	4	Nil	TMZ (4 cycles)	Partial	20m
DG14 (45F)	Feb05 Mar05	AA	7	XRT and TMZ, started before V1	Nil	N/A	9m
DG15 (39M)	Apr05	AA (rec)	9	Resection Aug00, post-operative XRT Resection Jan02, post-operative TMZ	TMZ (3 cycles)	Partial	16m

AA = anaplastic astrocytoma; GBM = glioblastoma multiforme; LGA = low grade astrocytoma; TMZ = temozolomide; XRT = external beam radiotherapy; rec = recurrent; m = months; V = vaccination.

Table 4.2 The clinical outcomes of using TMZ + monocyte-derived dendritic cells (DC) pulsed with autologous tumor cells (Hunn et al., 2014).

Patient	Vaccination Received (Prime + Boost)	Cycles of TMZ Received	Time to Progression (Months)	Overall Survival From Inclusion (Months)
A01	3+6	6	31.5	40
A02	3+3	4	5	7
A05	3+3	4	2	7
A06	3+5	4	6	10
A08	3+6	6	12.3	14
A09	3+2	2	3	7.8
A12	3+3	3	5.3	6
A13	3+2	2	2	7.8
A14	3+1	2	3.5	10.5

Table 4.3 Patients data and information about previous treatment (Hunn et al., 2014).

Patient	Age	Sex	Histology at first presentation	ECOG performance status at relapse	Cycles of TMZ completed at time of relapse	Time between last TMZ and relapse (months)	Location of recurrent tumour	Histology at re-operation
A01	39	M	GBM	1	6	1.5	R Frontal	GBM
A02	36	F	GBM	1	6	18	R Temp-Par	GBM
A03	64	M	GBM	1	6	0.5	R Temp	GBM
A04	69	M	GBM	1	5	0.5	R Insula	GBM
A05	57	F	GBM	1	6	3.5	R Fronto-Par	GBM
A06	39	M	GBM	2	6	6.5	L Frontal	GBM
A07	52	M	GBM	2	5	<1	R Frontal	GBM
A08	40	F	GBM	2	4	0.5	R Frontal	GBM
A09	30	F	GBM	0	6	0.5	R Frontal	GBM
A10	33	F	GBM	2	6	1	R Temp/BG	GBM
A11	50	F	GBM	2	6	<1	R Temp-Par	Necrosis[a]
A12	35	M	GBM	1	6	22	L Frontal/CC	GBM
A13	52	F	GBM	2	6	2	R Frontal	GBM
A14	65	M	GBM	2	6	3	L Frontal	GBM

R right, *L* left, *BG* basal ganglia, *CC* corpus callosum

[a] Patient withdrawn from trial

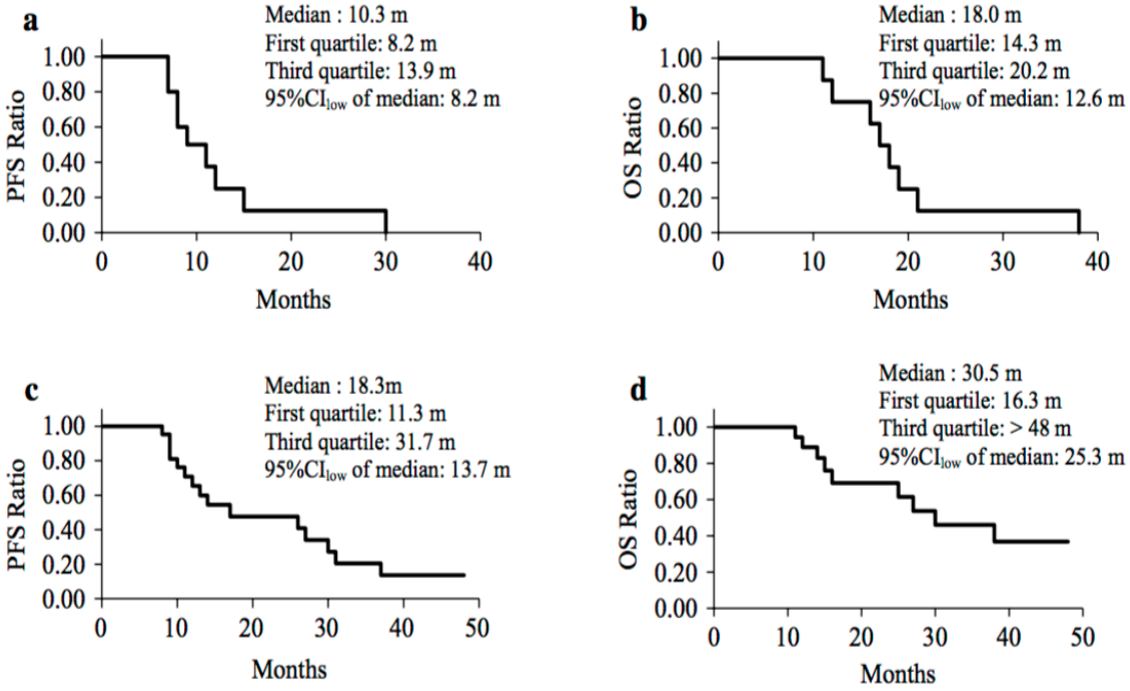

Figure 4.3 The Kaplan–Meier curves of **a** PFS and **b** OS of recurrent GBM patients, **c** PFS and **d** OS of newly diagnosed GBM patients after being treated with TMZ and FC (Akasaki et al., 2016)

The phase 1 clinical trial was conducted on 34 patients with malignant glioma and treated with autologous tumor lysate-pulsed DC (ATL-DC) (28 patients). The study demonstrated the absence of dose-limiting toxicity on GBM patients. Clinical outcomes which were expressed as PFS and OS values are shown in table 4.5 (Prins et al., 2013).

4.1.1.4 Clinical Outcomes of the Treatment Strategy of DC Vaccine loaded with Tumor Specific Antigens (SA) and Chemotherapy

ICT-107 is an autologous vaccine consisting of patient DC pulsed with six synthetic class I peptides (AIM-2, MAGE1, TRP-2, gp100, HER2/neu, and IL-13Ra2). These antigens are known tumor specific antigens and some of them are overexpressed on Cancer Stem Cells CSCs. A single-arm phase I study was conducted on 21 GBM patients whose tumors expressed at least three of these antigens. Patients with newly diagnosed and recurrent GBM who had their tumor resected and received a standard treatment of TMZ and RT were recruited. Patients were treated with ICT-107 vaccine to investigate its immunogenicity, safety and efficacy. After receiving DC based vaccination, patients with newly diagnosed GBM received TMZ and patients with recurrent GBM received TMZ with or without bevacizumab. Clinical outcomes were evaluated as PFS and OS in months as shown in table 4.4 (Phuphanich et al., 2012).

Median PFS in newly diagnosed patients was 16.9 months, and median OS was 38.4 months. 55.6 % of the treated patients reached three-year overall survival. The Kaplan–Meier probability curves of PFS and OS are shown in Figure 4.4 (Phuphanich et al., 2012).

Table 4.4 Clinical outcomes of ICT-107 vaccine on GBM patients (Phuphanich et al., 2012).

Patient ID	Age	Sex	Site	Extent of resection	Time to progression (months)	Survived time (months)	Immune response[b] IFNγ^{hi}, IFNγ^{lo}	Karnofsky score[d]
2	64	M	R temporal parietal	Complete	11.15	28.60	0.76, 0.65	100
4	46	M	L frontal	Complete	60.95[a]	60.95[a]	**1.74**, 1.15	90
6	56	F	L temporal	Complete	66.51[a]	66.51[a]	1.03, 0.96	100
7	61	F	R frontal	Complete	60.10[a]	60.10[a]	**3.58**[c]	90
9	51	M	R anterior temporal	Complete	6.87	19.53	0.67, 0.95	90
10	47	M	L frontoparietal	Complete	15.98	53.03[a]	1.49, 1.00	90
11	53	F	R temporal	Sub-total	12.62	25.91	0.61, 0.74	90
12	65	M	R temporal	Complete	8.25	22.55	0.43, 0.49	90
13	60	M	L temporal	Complete	29.0	38.37	**2.19**, 0.53	90
14	44	M	R temporoparietal	Complete	49.38*	49.38*	0.46, **1.73**	80
15	34	M	R parietal	Complete	48.66[a]	48.66[a]	0.43, 0.82	90
16	63	M	L parietal	Sub-total	17.72	32.42	1.07, 1.18	60
17	79	M	R frontal	Sub-total	11.28	15.98	0.62, **1.83**	70
18	52	F	Bifrontal	Complete	8.88	33.99	1.21, 1.01	90
19	48	M	L Frontal	Complete	47.64[a]	47.64[a]	0.56, 0.91	80
20	62	M	L Temporal	Sub-total	7.27	41.82[a]	NT	90

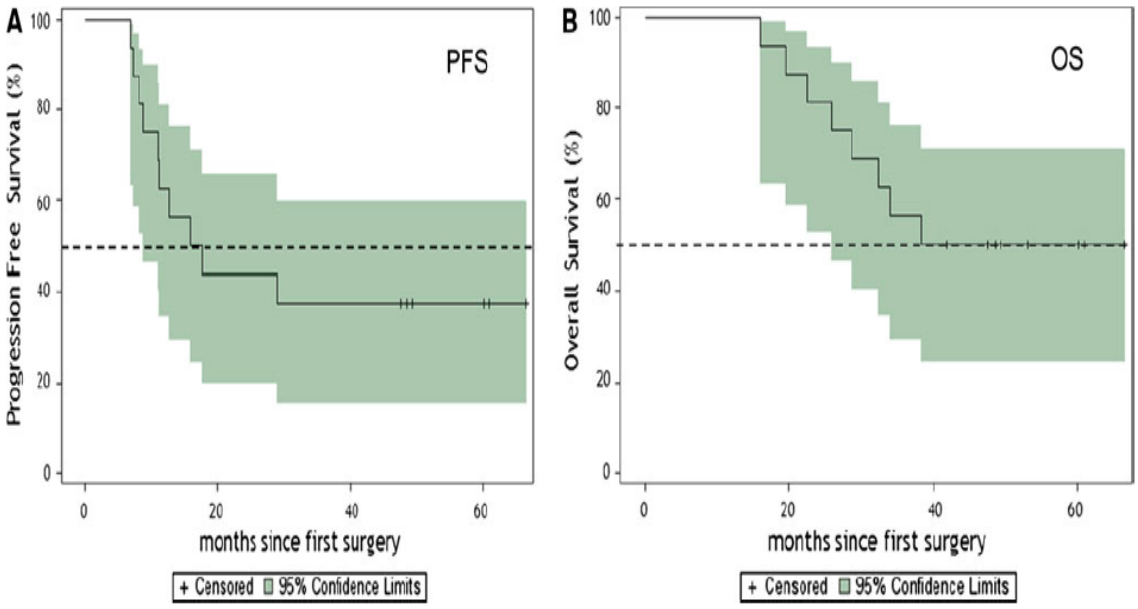

Figure 4.4 The Kaplan–Meier probability curves of (a) PFS and (b) OS of GBM patients (n=16) (Phuphanich et al., 2012).

The phase 2 clinical trial has been conducted to determine the safety, efficacy, and the ability to stimulate the immune response of 124 GBM patients of ICT-107 after surgery and chemotherapy. A phase 3 clinical trial is also under investigation (Clinicaltrials.gov, 2016).

The phase 1 clinical trial was conducted on 34 patients with malignant glioma and treated with glioma-associated antigen peptide-pulsed DC vaccination (GAA-DC) (six patients). The study demonstrated the absence of dose-limiting toxicity on GBM patients. The trial used the synthetic peptide antigens TRP-2, gp100, her-2/neu, and survivin which are known to be expressed by gliomas (Andersen et al., 2013; Zhang et al., 2008). Clinical outcomes which were expressed as PFS and OS values are shown in table 4.5 (Prins et al., 2013).

4.1.2 Immune Responses and Prognostic Biomarkers

Assays that measure the immune responses are crucial for the detection of anti-tumor responses that were developed after DC vaccine. Different tumor specimen analysis and assays such as the immunohistochemical analysis and CTL assays have been performed in clinical trials to determine immune responses that are strongly related to improvements in OS and PFS to identify new prognostic biomarkers.

Table 4.5 OS, PFS, and clinical characteristics of GBM patients who were either treated with ATL-DC (n = 28) or GAA-DC (n=6) (Prins et al., 2013).

Characteristic	**ATL-DC**	**GAA-DC**
	(N=28)	**(N=6)**
AGE – yr	49	44
Gender		
• Male	20	6
• Female	8	0
KPS (@ DC vacc.)	90	80
Tumor Pathology		
• Glioblastoma (WHO Grade IV)	23 (82.1)	4 (66)
• Anaplastic glioma (WHO Grade III)	5 (17.9)	2 (33)
Tumor Characteristics		
• IDH1 (% mutated)	17	50
Time to Treatment* **(months)**	4.9+/-4.1	4.4+/-1.8
Survival Characteristics		
• OS (months)	34.4	14.5
• PFS (months)	18.1	9.6

4.1.2.1 Tumor-Specific Cytotoxic T Lymphocyte CTL Responses

CTL assays were used to determine systemic tumor-specific cytotoxicity in GBM patients who received dendritic cell vaccine pulsed with acid-eluted tumor peptides as shown in figure 4.5. Patients who developed post-vaccination peripheral tumor-specific CTL activity have reached longer survival and did not experience progressive disease in contrast with patients who did not develop post-vaccination CTL.

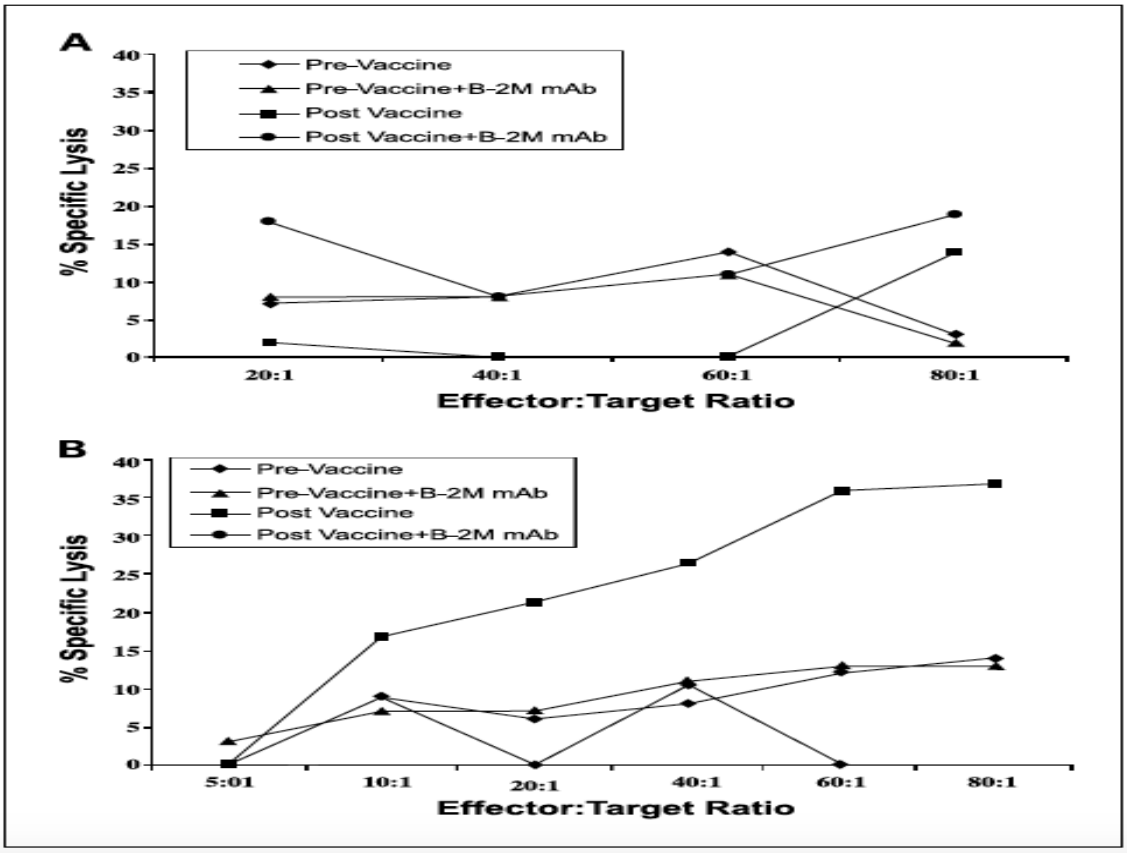

Figure 4.5 Peripheral CTL responses to autologous DC vaccine pulsed with acid-eluted tumor peptides. Negative CTL in patient (A) and positive CTL in patient (B) (Liau et al., 2005).

The up-regulation of several glioma-associated antigens such as WT-1 (M. Y. Chen et al., 2010), gp-100 (Xie, Nguyen, Hupe, & Wei, 2009), and MAGE-A3 (Monte et al., 2006) makes the tumor cells resistant to chemotherapy. These antigens are known as chemo resistance associated peptides (CAPs). The assessment of CTL responses against these TAAs was performed to investigate the correlating with OS as shown in figure 4.6. Antigen-specific CTL responses against WT-1 (a), gp-100 (b), and MAGE-A3 (c) were detected in four patients who reached the longest OS after vaccination with FCs.

The OS was 17.8 months in patient 1, 21.2 months in patient 2 , > 36 months in patient 3, and reached > 48 months in patient 4 (Akasaki et al., 2016) .

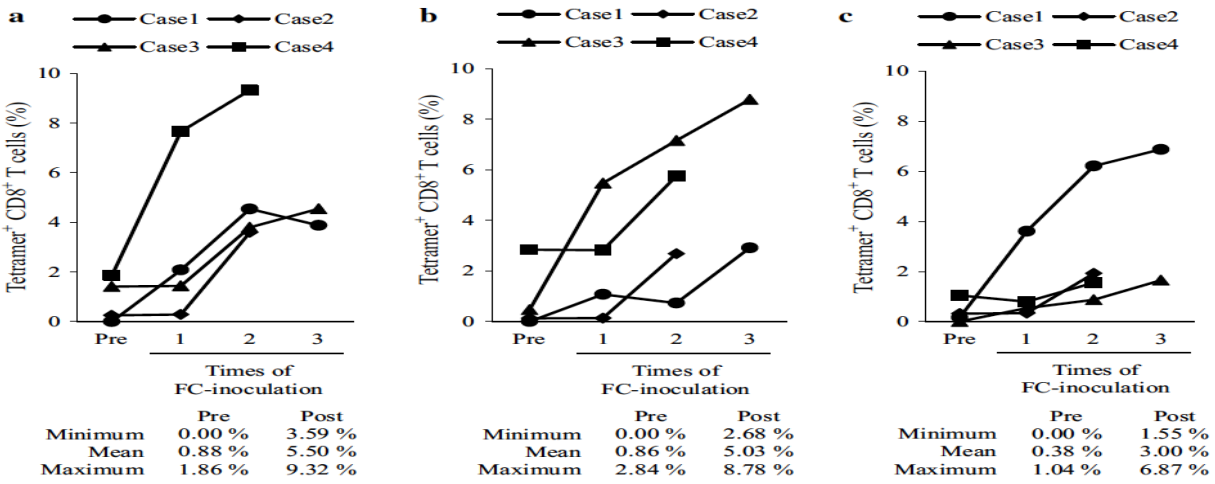

Figure 4.6 Expression of WT-1, gp-100, and MAGE-A3 in 4 GBM patients (Akasaki et al., 2016).

4.1.2.2 CD3$^+$, Tumor-Infiltrating Lymphocytes (TIL), and Transforming Growth Factor-β2 TGFβ2

Immunohistochemical analysis of tumor specimens from the pre-vaccination and post-vaccination surgery has been performed. Cytotoxic T cells CD8$^+$ and memory T cells (CD45RO) infiltrates were found to be elevated in all post-vaccination specimens compared with the pre-vaccination specimen. The elevation of cytotoxic T cells CD8$^+$ after vaccination is shown in figure 4.7 (Walker et al., 2008).

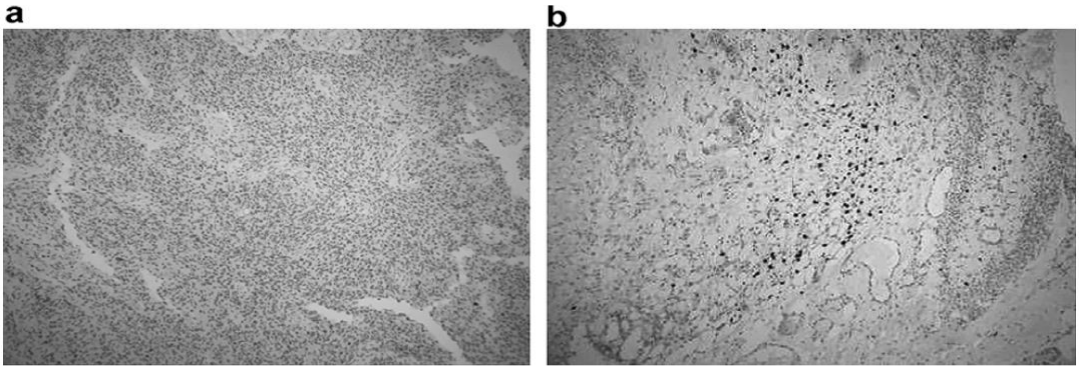

Figure 4.7 Cytotoxic T cells CD8+ (dark cells) in malignant astrocytoma tissue before DC vaccination (a), and after DC vaccination (b) (Walker et al., 2008) .

Patients with robust infiltration of $CD3^+$ and tumor-infiltrating lymphocytes (TIL) such as $CD8^+$, $CD45RO^+$ memory T cells, and $CD4^+$ helper T cells reached >30 months OS as shown in figure 4.9 (A and B). Those patients also had lower expression of transforming growth factor-β2 TGFβ2 in their GBM cells samples as shown in figure 4.8. The accumulation of TGF-β2 within the tumor microenvironment may result in the absence TIL infiltration and a clinically significant antitumor immune response in GBM patients. Patients with no significant difference in the infiltration of TIL as shown in figure 4.9 (C) have experienced tumor progression and died within 1 year. Figure 4.9 (D) also shows a control group of GBM patients who did not receive DC vaccination (Liau et al., 2005). A recent study also showed a significant correlation between high TIL content and increased OS and PFS (Sedighim et al., 2016).

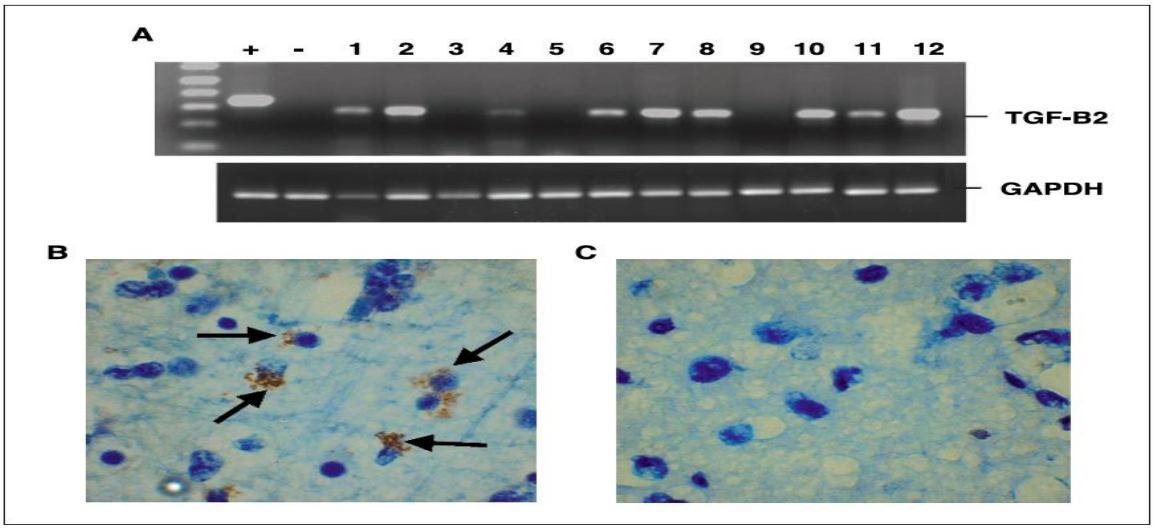

Figure 4.8 TGF-β2 expressions in GBM specimens. Analysis of TGF-β2 mRNA (A), high TGF-β2 (B), low TGF-β2 protein expression (C) in GBM tissue (Liau et al., 2005).

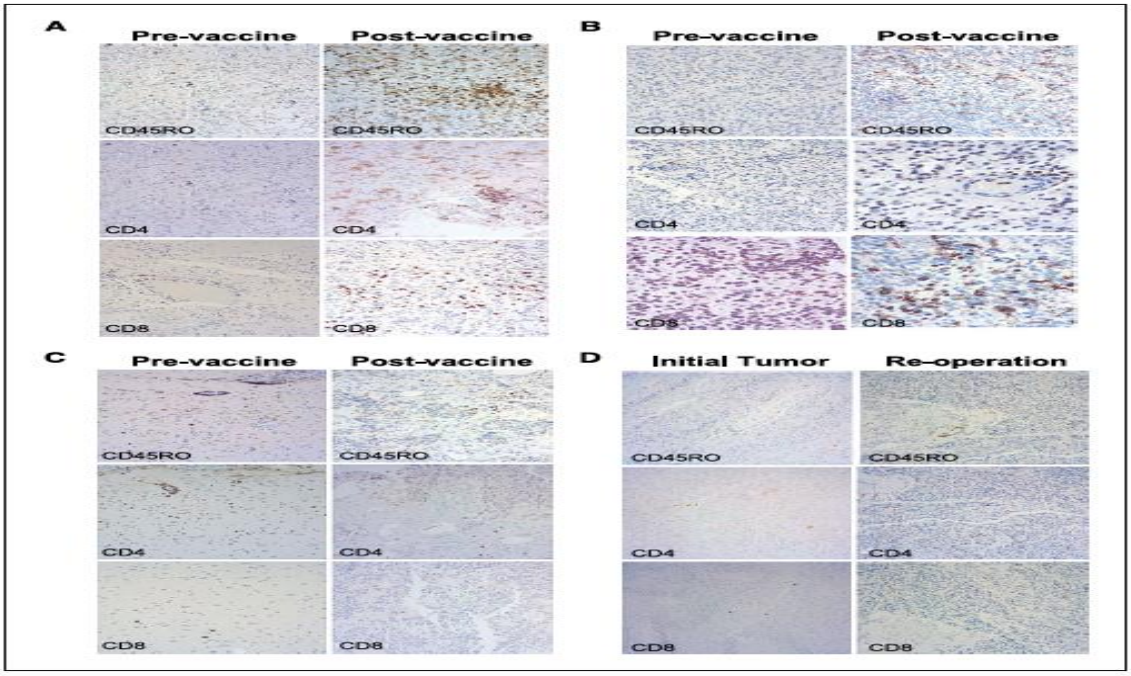

Figure 4.9 Infiltration of TIL into GBM cells after receiving DC vaccine pulsed with acid-eluted tumor peptides (Liau et al., 2005).

4.1.2.3 Immune Responses to Specific TAAs

Patients with optimum clinical outcomes (A01, A08) experienced baseline immune responses to antigens in the tumor material as well as responses to TRP-2, survivin and SOX-2 (Hunn et al., 2014). The two patients also had the lowest level of Treg at baseline and one of these also had the lowest level of putative Myeloid-Derived Suppressor Cells MDSC and high proportion of $CD8^+$. One patient (A08) also had an unusually high proportions of $CD4^+$ effector cells. Response categorization was based on the WHO UICC system (Hunn et al., 2014).

A significant correlation of PFS and OS with quantitative expression of MAGE1 and AIM-2 were detected. Patients with overexpression of HER2 and gp100 reached a relatively long survival (Phuphanich et al., 2012).

4.1.2.4 CD133 Expression

A decrease in or absence of CD133 expression was found in five patients who underwent a second resection. Patient (#03) experienced recurrent GBM and a decrease in CD133 expression after vaccination as shown in Figure 4.10. Patient (#08) had negative expression of CD133. Patient (#10) with a newly diagnosed disease was negative for CD133 in both the primary and recurrent tumor. Another patient (# 09) experienced a one-log decrease in CD133 expression. Patient (#19) was negative for CD133 in the second surgical sample and did not experience a progressive disease (Phuphanich et al., 2012).

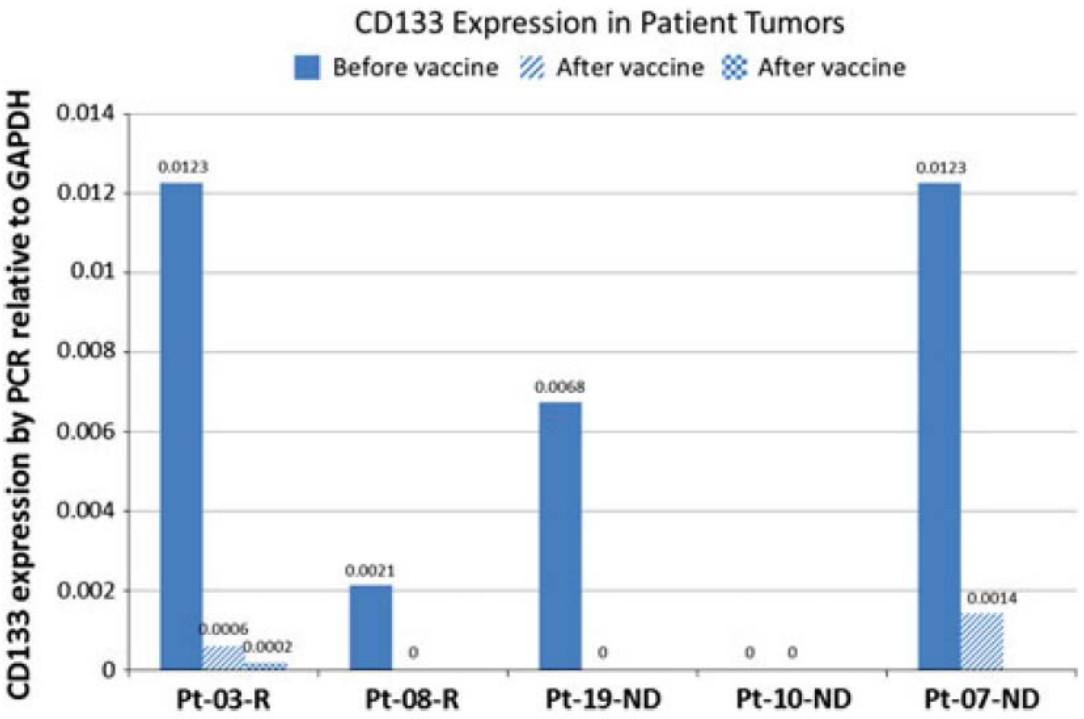

Figure 4.10 CD133 expression after vaccination with ICT-107 (Phuphanich et al., 2012).

4.1.2.5 The Inhibition of Anti-Tumor Immune Responses by Treg and NK

The GAA-DC trial have been encountered regulatory T cell or NK cell populations that inhibited anti-tumor immune responses. NK cells activated populations $CD3^-$, $CD16^+$, $CD25^+$ were found to be significantly increased in the peripheral blood of patients received GAA-DC. A remarkable independent association between Treg cell ratios (post/pre-DC vaccination) and overall survival was also reported as shown in table 4.6. The inhibition of Treg and activated NK cells in GBM patients following DC vaccination was accompanied by observed extended survival. Based on these findings.

Treg ratio post/pre-DC vaccination may be considered as prognostic biomarkers for OS in GBM patients (Prins et al., 2013).

Table 4.6 Stratified Cox proportional hazards model for survival with clinical endpoints and immune monitoring ratios (Prins et al., 2013).

Covariate*	Hazard Ratio	95% C.I. for Hazard Ratio	p-value
Age (1 unit increase in years)	1.03	(0.99, 1.08)	0.187
Gender (Female vs Male)	1.77	(0.51, 6.10)	0.368
KPS	0.92	(0.86, 0.98)	0.010
Overall Tumor Path Effects			0.023
Recurrent Grade IV vs. newly dx Grade IV	4.42	(1.46, 13.38)	0.009
Recurrent Grade IV vs. Grade III	6.86	(0.62, 75.91)	0.116
Grade IV vs. Grade III	1.55	(0.15, 15.56)	0.709
Treg cell fold change**	7.19	(1.87, 27.73)	0.004
Activated NK cell fold change**	1.99	(0.92, 4.31)	0.081

*Each model includes a single covariate. Stratification is on trials.

**Refers to frequency of cells (%) at post-DC vaccination/pre-DC vaccination

4.1.2.6 The Effect of TMZ + RT Treatment Strategy on Immune Response

TMZ + RT treatment strategy was reported to selectively reduce $CD4^+$ T cells which may result in the reduction of their negative effects on immune therapy. TMZ was also reported to cause depletion of $CD4^+CD25^+$ T-cell subsets (Su et al., 2004). The identified Treg cells suppress T-cell responses and the depletion of these cells has resulted in a significant enhancement of $CD8^+$ T-cell immunity in animal models (Sakaguchi et al., 2001; Mischo Kursar et al., n.d.).

4.1.2.7 DC Migration to the Draining Lymph Nodes

A recent study showed that pre-conditioning the vaccine site with a potent recall antigen such as tetanus/diphtheria (Td) toxoid has been resulted in a significant enhancement of the lymph node homing, DC migration bilaterally, and improved OS. There was a significant accumulation of injected DCs in vaccine site draining lymph nodes (VDLNs) in patients who received Td in contrast with patients who received un-pulsed DCs as shown in Figure 4.11 (a). Moreover, patients received Td achieved a significant increase in both PFS as shown in figure 4.11 (b) and OS as shown in figure 4.11 (c) in contrast with the other group of patients. These findings suggest that DC migration should be considered as a predictive biomarker for DC-base vaccination studies as well as other immunotherapy studies (Mitchell et al., 2015).

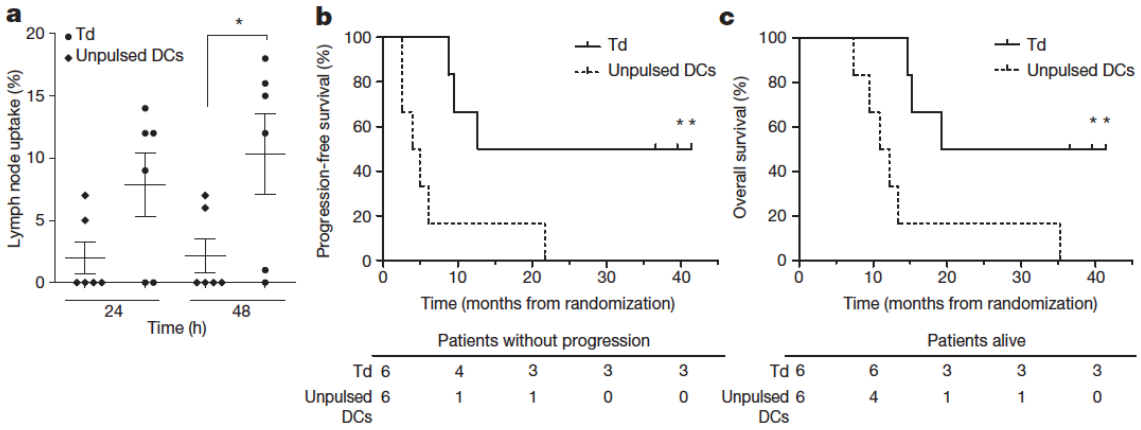

Figure 4.11 Td pre-conditioning enhances DC migration to VDLNs and increase OS and PFS (Mitchell et al., 2015).

4.2 Data Analysis and Findings

Patients' information, the inclusion criteria, pre and post vaccination treatment strategies, and clinical outcomes (OS and PFS) in published clinical trials are shown in table 4.7.Independent samples t-tests have been used to compare the means of OS and PFS values of independent sample groups of GBM patients. Moreover, ANOVA test has been performed to compare the means of OS and PFS values of two or more independent sample groups of GBM patients.

The first independent samples t-test has been performed to determine the presence of a statistically significant difference of OS and PFS values at 95% confidence level ($\alpha = 0.05$) between two groups of GBM patients. The first group have received DC based vaccination pulsed with specific antigens (SA) with concomitant or post vaccination TMZ (n=22 for comparing OS and PFS). The sources of SA antigens include the synthetic class I peptides (AIM-2, MAGE1, TRP-2, gp100, HER2/neu, and IL-13Ra2) on 21 patients or the synthetic peptides (TRP-2, gp100, her-2/neu, and survivin) on six patients. The second group of GBM patients received DC based vaccination loaded with tumor material antigens (TM) and concomitant or post vaccination TMZ (n=78 for OS and n= 69 for PFS). The sources of TM antigens include autologous tumor or glioma cells. The null hypothesis is H0: $\mu_{SA} = \mu_{TM}$ and the alternative hypothesis is H1: $\mu_{SA} > \mu_{TM}$.Test results showed that with 95% confidence level the mean of the OS and PFS values for the group of patients who received DC based vaccination pulsed with specific antigens with concomitant or post vaccination TMZ (SA) is higher than the mean of OS and PFS values of the group of patients who received DC--Vaccination loaded with

tumor material antigens and concomitant or post vaccination TMZ (TM) (p= 0.006 and 0.002 respectively).

Table 4.7 Patients' information, KFS status, antigen sources, treatment strategies, OS, and PFS values.

Patient	Antigen Source	Pre-Vaccination TMZ	Concomitant or post-vaccination TMZ	Age (years)	KPS	OS (months)	PFS (months)	References
A01	Tumor Material	Nil	TMZ (2 cycles)	55	≥ 70 %	12	-	Walker et al., 2008
A02	Tumor Material	Nil	Nil	71	≥70%	2	-	
A03	Tumor Material	Nil	Nil	25	≥70%	4	-	
A04	Tumor Material	Nil	Nil	52	≥70%	5	-	
A05	Tumor Material	Nil	TMZ (6 cycles)	44	≥70%	18	-	
A06	Tumor Material	Nil	Nil	66	≥70%	9	-	
A07	Tumor Material	Nil	TMZ	47	≥70%	15	-	
A08	Tumor Material	Nil	TMZ	60	≥70%	11	-	
A09	Tumor Material	Nil	TMZ (2 cycles)	55	≥70%	5	-	
A10	Tumor Material	TMZ (6 cycles)	TMZ (6 cycles)	39	≥80%	40	31.5	Hunn et al., 2014
A11	Tumor Material	TMZ (6 cycles)	TMZ (4 cycles)	36	≥%80	7	5	
A12	Tumor Material	TMZ (6 cycles)	TMZ (4 cycles)	57	≥80%	7	2	
A13	Tumor Material	TMZ (6 cycles)	TMZ (4 cycles)	39	≥70%	10	6	
A14	Tumor Material	TMZ (6 cycles)	TMZ (6 cycles)	40	≥70%	14	12.3	
A15	Tumor Material	TMZ (4 cycles)	TMZ (2 cycles)	30	100%	7.8	3	
A16	Tumor Material	TMZ (6 cycles)	TMZ (3 cycles)	35	≥80%	6	5.3	
A17	Tumor Material	TMZ (6 cycles)	TMZ (2 cycles)	52	≥70%	7.8	2	
A18	Tumor Material	TMZ (6 cycles)	TMZ (2 cycles)	65	≥70%	10.5	3.5	

Table 4.7 continued

Patient	Antigen Source	Pre-Vaccination TMZ	Concomitant or post-vaccination TMZ	Age (years)	KPS	OS (months)	PFS (months)	References
A19	Specific Antigens	TMZ + RT	TMZ	64	100%	28.6	11.5	Phuphanich et al., 2012
A20	Specific Antigens	TMZ + RT	TMZ	46	90%	60.95	60.95	
A21	Specific Antigens	TMZ + RT	TMZ	56	100%	66.51	66.51	
A22	Specific Antigens	TMZ + RT	TMZ	61	90%	60.1	60.1	
A23	Specific Antigens	TMZ + RT	TMZ	51	90%	19.53	6.87	
A24	Specific Antigens	TMZ + RT	TMZ	47	90%	53.03	15.98	
A25	Specific Antigens	TMZ + RT	TMZ	53	905	25.91	12.62	
A26	Specific Antigens	TMZ + RT	TMZ	65	90%	22.55	8.25	
A27	Specific Antigens	TMZ + RT	TMZ	60	90%	38.37	29	
A28	Specific Antigens	TMZ + RT	TMZ	44	≥80%	49.38	49.38	
A29	Specific Antigens	TMZ + RT	TMZ	34	90%	48.66	48.66	
A30	Specific Antigens	TMZ + RT	TMZ	63	≥60%	32.42	17.72	
A31	Specific Antigens	TMZ + RT	TMZ	79	≥70%	15.98	11.28	
A32	Specific Antigens	TMZ + RT	TMZ	52	90%	33.99	8.88	
A33	Specific Antigens	TMZ + RT	TMZ	48	≥80%	47.64	47.64	
A34	Specific Antigens	TMZ + RT	TMZ	62	90%	41.82	7.27	
A35	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	Prins et al., 2013
A36	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A37	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A38	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	

Table 4.7 continued

Patient	Antigen Source	Pre-Vaccination TMZ	Concomitant or post-vaccination TMZ	Age (years)	KPS	OS (months)	PFS (months)	References
A39	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A40	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A41	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A42	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A43	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A44	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A45	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A46	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A47	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A48	Tumor material	TMZ + RT	TMZ	49	905	34.4	18.1	
A49	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A50	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A51	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A52	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A53	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A54	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A55	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A56	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A57	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A58	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	

Table 4.7 continued

Patient	Antigen Source	Pre-Vaccination TMZ	Concomitant or post-vaccination TMZ	Age (years)	KPS	OS (months)	PFS (months)	References
A59	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A60	Tumor material	TMZ + RT	TMZ	49	905	34.4	18.1	
A61	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A62	Tumor material	TMZ + RT	TMZ	49	90%	34.4	18.1	
A63	Specific Antigens	TMZ + RT	TMZ	44	≥80%	14.5	9.6	
A64	Specific Antigens	TMZ + RT	TMZ	44	≥80%	14.5	9.6	
A65	Specific Antigens	TMZ + RT	TMZ	44	≥80%	14.5	9.6	
A66	Specific Antigens	TMZ + RT	TMZ	44	≥80%	14.5	9.6	
A67	Specific Antigens	TMZ + RT	TMZ	44	≥80%	14.5	9.6	
A68	Specific Antigens	TMZ + RT	TMZ	44	≥80%	14.5	9.6	
A69	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	Akasaki et al., 2016
A70	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	
A71	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	
A72	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	
A73	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	
A74	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	
A75	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	
A76	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	
A77	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	
A78	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	

Table 4.7 continued

Patient	Antigen Source	Pre-Vaccination TMZ	Concomitant or post-vaccination TMZ	Age (years)	KPS	OS (months)	PFS (months)	References
A79	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	
A80	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	
A81	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	
A82	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	
A83	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	
A84	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	
A85	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	
A86	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	
A87	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	
A88	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	
A89	Tumor material	Nil	TMZ	54.6	≥ 50 %	30.5	18.3	
A90	Tumor material	Nil	TMZ	54.6	≥ 50 %	18	10.3	
A91	Tumor material	TMZ	TMZ	54.6	≥ 50 %	18	10.3	
A92	Tumor material	TMZ	TMZ	54.6	≥ 50 %	18	10.3	
A93	Tumor material	TMZ	TMZ	54.6	≥ 50 %	18	10.3	
A94	Tumor material	TMZ	TMZ	54.6	≥ 50 %	18	10.3	
A95	Tumor material	TMZ	TMZ	54.6	≥ 50 %	18	10.3	
A96	Tumor material	TMZ	TMZ	54.6	≥ 50 %	18	10.3	
A97	Tumor material	TMZ	TMZ	54.6	≥ 50 %	18	10.3	
A98	Tumor material	TMZ	TMZ	54.6	≥ 50 %	18	10.3	
A99	Tumor material	TMZ	TMZ	54.6	≥ 50 %	18	10.3	
A100	Tumor material	TMZ	TMZ	54.6	≥ 50 %	18	10.3	

There is a statistically significant difference of the means of OS and PFS values between the two groups. DC vaccine pulsed with specific antigen that were found to be overexpressed on tumor cells of patients achieved more beneficial clinical outcome than DC vaccines loaded with tumor material.

ANOVA test has been performed to compare the means of OS and PFS values of the two groups of GBM patients as shown in figure 4.12 and figure 4.13.

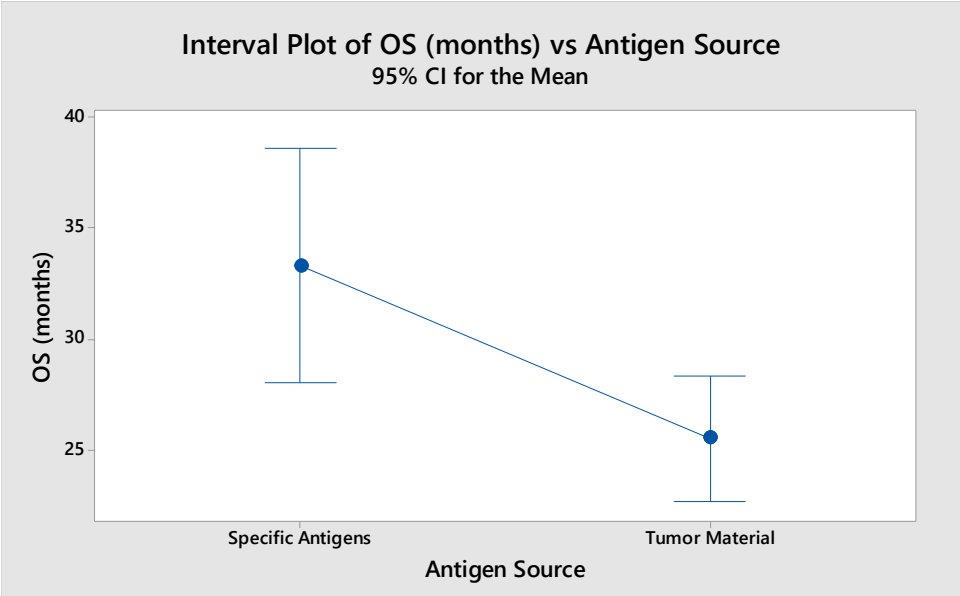

Figure 4.12 The difference in the means of OS values between two groups of GBM patients.

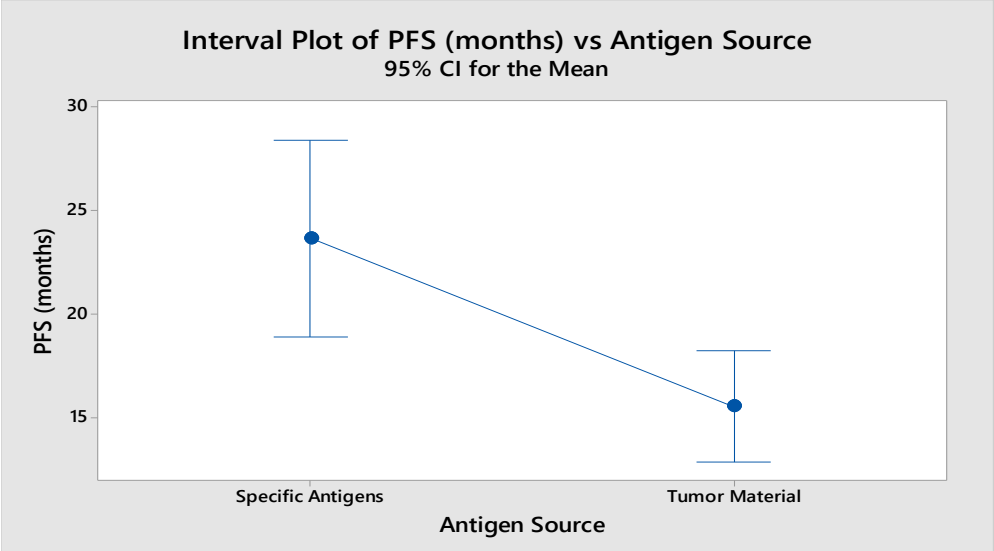

Figure 4.13 The difference in the means of PFS values between two groups of GBM patients.

The second independent samples t-test has been performed to determine the presence of a statistically significant difference of OS and PFS values at 95% confidence level ($\alpha = 0.05$) between two groups of GBM patients. The first group have received DC vaccine + concomitant or post vaccination TMZ and did not receive pre-vaccination TMZ (Nil) (n=31 for OS and n= 22 for PFS). The second group of GBM patients received pre-vaccination TMZ + DC vaccine + concomitant or post-vaccination TMZ (TMZ) (n= 19 for OS and PFS). The null hypothesis is H0: $\mu_{Nil} = \mu_{TMZ}$ and the alternative hypothesis is H1: $\mu_{Nil} > \mu_{TMZ}$.

Test results showed that with 95% confidence level (α=.05), the means of the OS and PFS values for the group of patients who received DC vaccine + concomitant or post vaccination TMZ and did not receive pre-vaccination TMZ (Nil) is higher than the mean of OS and PFS values of the group of patients who received pre-vaccination TMZ + DC vaccine+ concomitant or post-vaccination TMZ (P-Value = 0.001 and 0.000 respectively). There is a statistically significant difference of the means of OS and PFS

values between the two groups. Our result suggested that receiving pre vaccination TMZ had been resulted in less beneficial clinical outcomes.

ANOVA test has been performed to compare the means of OS and PFS values of the two groups of GBM patients as shown in figure 4.14.

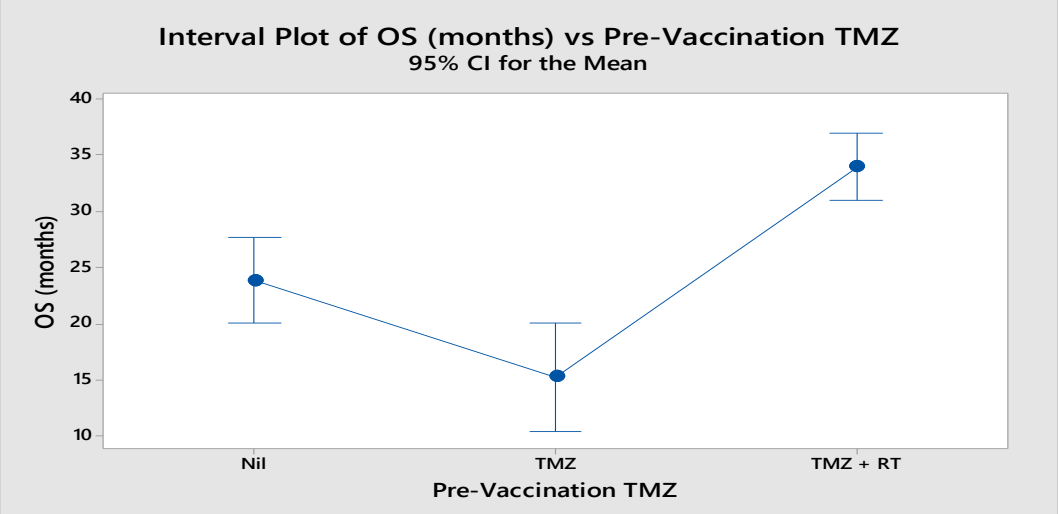

Figure 4.14 The difference in the means of OS values between different groups of GBM patients.

The third independent samples t-test has been performed to determine the presence of a statistically significant difference of OS values at 95% confidence level ($\alpha = 0.05$) between two groups of GBM patients. The first group received pre-vaccination TMZ + RT + DC vaccine + concomitant or post-vaccination TMZ (TMZ+RT, n=50). The second group of GBM patients DC vaccine + concomitant or post vaccination TMZ and did not receive pre-vaccination therapy (Nil) (n=31). The null hypothesis is H0: $\mu_{TMZ+RT} = \mu_{Nil}$ and the alternative hypothesis is H1: $\mu_{TMZ+RT} > \mu_{Nil}$.

Test results showed that with 95% confidence level (α=0.05), the mean of the OS values for the group of patients who received pre-vaccination TMZ + RT + DC vaccine + concomitant or post-vaccination TMZ (TMZ+RT) is higher than the mean of OS values for the group of patients who received DC vaccine + concomitant or post vaccination TMZ and did not receive pre-vaccination therapy (Nil) (P-Value = 0.000). There is a statistically significant difference of the means of OS values between the two groups. Receiving pre vaccination TMZ+RT achieved relatively more OS than receiving pre vaccination Nil as shown in figure 4.14.

The fourth independent samples t-test has been performed to determine the presence of a statistically significant difference of PFS values at 95% confidence level (α = 0.05) between two groups of GBM patients. The first group received pre-vaccination TMZ + RT + DC vaccine + concomitant or post-vaccination TMZ (TMZ+RT, n=50). The second group of GBM patients received DC vaccine + concomitant or post vaccination TMZ and did not receive pre-vaccination therapy (Nil, n=22). The null hypothesis is H0: $\mu_{TMZ+RT} = \mu_{Nil}$ and the alternative hypothesis is H1: $\mu_{TMZ+RT} > \mu_{Nil}$.

Test results showed that with 95% confidence level (α=.05), the mean of the OS values for the group of patients who received pre-vaccination TMZ + RT + DC vaccine + concomitant or post-vaccination TMZ is not higher than the mean of OS values for the group of patients who received DC vaccine + concomitant or post vaccination TMZ and did not receive pre-vaccination therapy (Nil) (P-Value = 0.194). There is no statistically significant difference of the means of the PFS values between the two groups.

ANOVA test has been performed to compare the means of PFS values of the two groups of GBM patients as shown in figure 4.15.

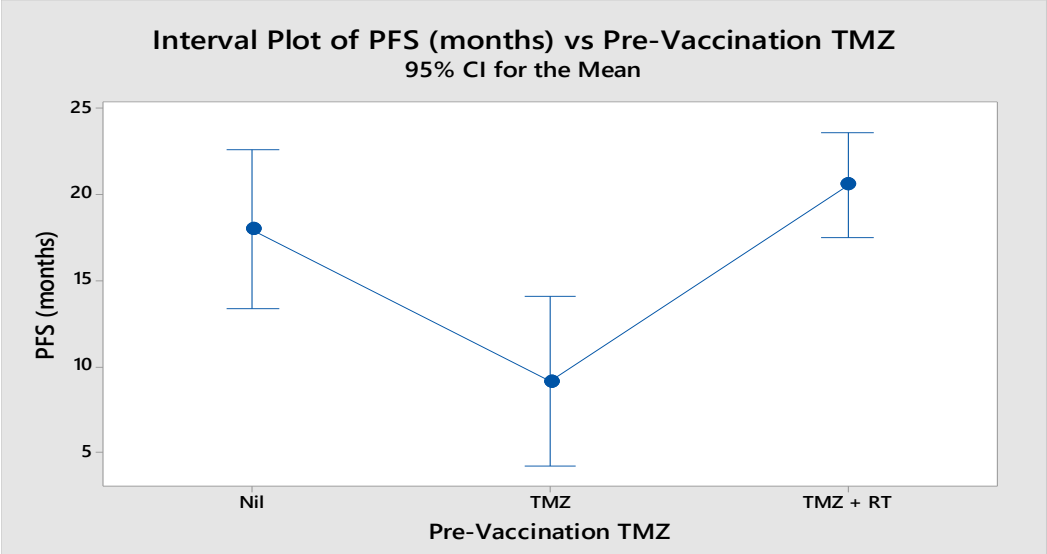

Figure 4.15 The difference in the means of the PFS values between different groups of GBM patients involved in different treatment strategies.

ANOVA test has been performed with 95% confidence level (α= 0.05) to determine the presence of a statistically significant difference between the means of OS and PFS values of two groups of GBM patients. The first group includes patients who received DC based vaccination + concomitant or post-vaccination TMZ with or without RT (n=100 for OS values and n=91 for PFS values). The second group includes patients who received TMZ+RT with no DC vaccine (n=287). The null hypothesis is H0: $\mu 1 = \mu 2$ and the alternative hypothesis is H1: $\mu 1 \neq \mu 2$.

The test results showed that there is a statistical significant difference between the means of OS (P=0.00) and PFS values (P=0.00) of the two groups of GBM patients as shown in figure 4.16 and figure 4.17.

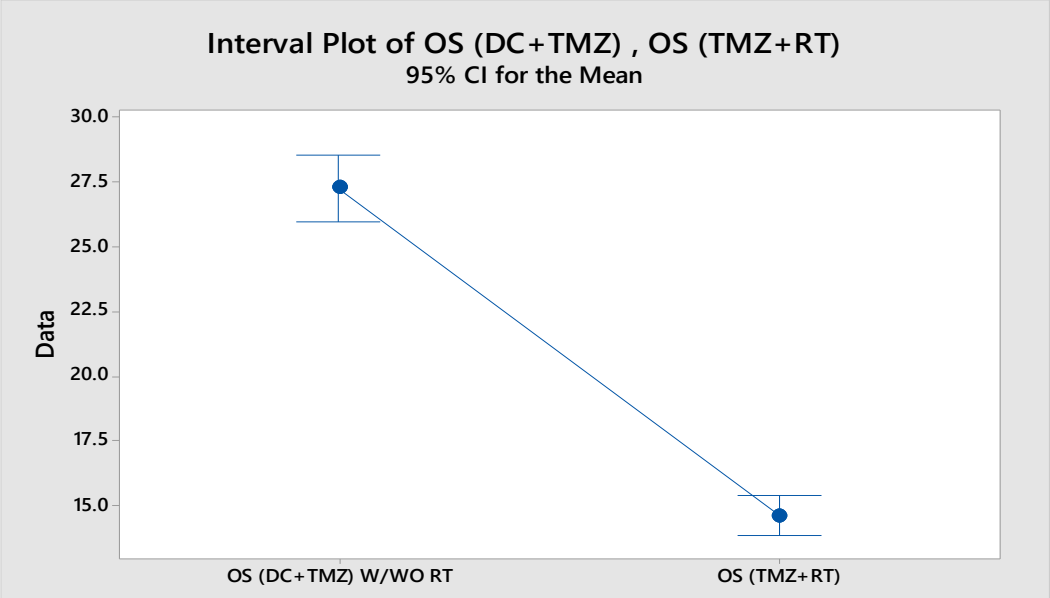

Figure 4.16 The difference of means of OS values between two groups of GBM patients.

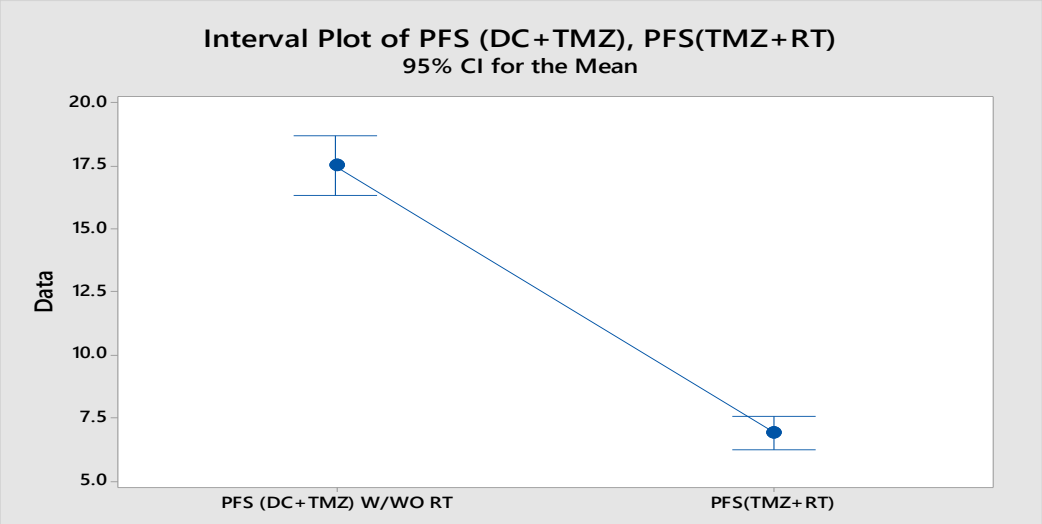

Figure 4.17 The difference of means of PFS values between two groups of GBM patients.

4.3 Summary

This chapter has presented the collected data, data analysis techniques, test results and findings of this research.

CHAPTER 5. DISCUSSION, CONCLUSION AND RECOMMENDATIONS

This chapter discusses the FDA's thinking of cancer vaccines, the conclusion and the results of this study and the recommendations for the future studies.

5.1 Discussion

5.1.1 FDA's Current Thinking on Investigational Studies of Cancer Vaccines

Chemotherapy and radiotherapy are immunosuppressive and may minimize the potential responsiveness to the cancer vaccine being tested on patients with recurrent diseases. The immunomodulatory effects of concomitant treatments such as chemotherapies should be justified and considered in the clinical trial design.

Anti-tumor immune response generally requires 2-3 months to be detected and initiate the effectiveness of a cancer vaccine. Cancer vaccines are better to be tested in patients with no evidence of residual disease to provide enough time for the cancer vaccine to initiate an immune response that could be measured. As a result, patients may experience early progression followed by subsequent response, which should be considered in the design of clinical trials.

The disadvantage of the clinical development of cancer vaccines is that it may require more patients and time than developing conventional therapies. This approach may not be able to provide interpretable evidence of efficacy because of recruiting patients with heterogeneous tumor types and stages. The preparation of vaccines using autologous patient materials is another challenge because of the differences in each patient and tumor histology. Heterogeneity of the patient population should be considered before selecting the patient population (Guidance for Industry Clinical Considerations for Therapeutic Cancer Vaccines, 2011).

5.1.2 FDA's Recommendations for Monitoring the Immune Response

Assays that measure the immune responses are crucial for the detection of anti-tumor responses that were developed after DC vaccine. At least two immunological assays should be used to determine the targeted anti-tumor response. Assay conditions, sensitivity and specificity of the assay, positive and negative controls, end points for the assessment of results, and the statistical analytical methods should be considered as important parameters and identified before starting clinical trials (Guidance for Industry Clinical Considerations for Therapeutic Cancer Vaccines, 2011).

5.2 Conclusion and Research Results

Current treatment options including surgery, radiotherapy, and temozolomide chemotherapy are associated with limited clinical benefits. The median OS reached by GBM patients was approximately 12 months only after being treated with radiotherapy alone without temozolomide. However, the median OS has been estimated as 14.6

months in patients who received the combined treatment of radiotherapy and chemotherapy with temozolomide.

There is a great need for an optimized therapy for the treatment of GBM. Dendritic Cells DCs are the most potent antigen presenting APCs in the immune system. DCs have the ability to capture and process neoantigens; which are formed and released by oncogenesis. DCs present the captured antigens on Histocompatibility Complex I and II (MHCI and MHCII) molecules to stimulate the native T cells and induce primary immune responses and peripheral immunological tolerance. After T cell responses against the cancer-specific antigens are primed and activated, T cells infiltrate the tumor bed, specifically recognize, bind to, and kill their target cancer cell. Clinical trials showed that treating GBM patients with surgery, TMZ, and Dendritic Cell DC vaccines was safe and achieved meaningful clinical outcomes. Different clinical outcomes and immune responses were associated with DC- based vaccination therapy. The sources of antigen that have been used in DC immunotherapy include synthetic class I peptides AIM-2, MAGE1, TRP-2, gp100, HER2/neu, and IL-13Ra2, synthetic peptides TRP-2, gp100, her-2/neu, and surviving, and autologous tumor or glioma cells.

Our study showed that the treatment strategy of DC-based vaccination combined with chemotherapy with or without RT has proved to achieve substantial clinical benefits. The median of OS and PFS of GBM patients who received DC vaccines combined with conventional treatments were 27.5 months and 17.5 months respectively. However, the median of OS and PFS of GBM patients who received conventional treatments of chemotherapy with TMZ and radiation therapy were 14.6 months and 6.9 months respectively.

This research also has showed a proof of evidence that clinical outcomes of different DC- based vaccination therapy for the treatment of GBM patients vary based on the sources of antigens and different pre-vaccination strategies. DC vaccines were loaded with Tumor Material, pulsed with different Tumor Associated Antigens TAAs, and combined with different pre and post vaccination treatment strategies. These variables resulted in different clinical outcomes and different levels of immune responses. DC vaccine pulsed with specific synthetic antigens that were found to be expressed in tumor cells of GBM patients achieved more beneficial clinical outcome than DC vaccines loaded with tumor material. Receiving pre vaccination TMZ has resulted in less beneficial clinical outcomes (OS and PFS) than receiving DC vaccine without pre-vaccination TMZ. Receiving pre vaccination TMZ+RT achieved relatively longer OS than receiving DC vaccine without pre-vaccination TMZ. However, there was no evidence of a meaningful improvement of PFS with the pre-vaccination treatment strategy of TMZ+RT.

Assays that measure the immune responses are crucial for the detection of anti-tumor responses that were developed after vaccination with DCs. Different tumor specimens' analysis and assays such as the immunohistochemical analysis and CTL assays have been performed in clinical trials to determine immune responses that were strongly related to the relatively long OS and PFS. There were many immune responses that were related to the most beneficial clinical outcomes; which can be considered as prognostic biomarkers of GBM. Patients who developed post-vaccination peripheral tumor-specific CTL activity have reached longer survival and did not experience progressive disease in contrast with patients who did not develop post-vaccination CTL.

Robust infiltration of $CD3^+$ and tumor-infiltrating lymphocytes (TIL) such as $CD8^+$, $CD45RO^+$ memory T cells, and $CD4^+$ helper T cells in post-vaccination specimens was found to be related to long OS. Antigen-specific CTL responses against specific antigens such as WT-1, gp-100, MAGE-A3, TRP-2, survivin and SOX-2 were also found to be related to a relatively long OS after vaccination with DC-based vaccination treatment. Lower expression of transforming growth factor-β2 TGFβ2 in GBM patients was found to be related to long OS. low level of Treg and putative Myeloid-Derived Suppressor Cells MDSC at baseline was found to be related to beneficial clinical outcomes. Moreover, the enhancement of DCs migration to the draining lymph nodes was also associated with a significant increase in both PFS and OS. Treg ratio post/pre-DC vaccination, DC migration to the draining lymph nodes, and the expression of TGFβ2, MDSC, and CD133 should be considered as prognostic biomarkers for DC-based vaccination studies.

The expression of the stem cell marker CD133 and specific Tumor Associated Antigens TAAs such as WT-1, gp-100, and MAGE-A3 on GBM cells was found to result in resistance to RT and chemotherapy and significant increase in tumor aggressiveness. Cytomegalovirus phosphoprotein 65pp65 HCMV was also found to be expressed in more than 90% of GBM specimens in contrast with normal brain. CD133, WT-1, gp-100, MAGE-A3, and HCMV viral proteins may be used as tumor-specific targets.

5.3 Future Recommendations

Future studies should investigate the rate-limiting steps that negatively affect the production of antitumor immune responses. Overcoming this problem will enhance the

effectiveness of DC vaccines and other immunotherapies. As we previously mentioned in the data analysis section, loading DCs with specific antigens that were found to be overexpressed in GBM cells has achieved substantial clinical outcomes. There is a great need to identify a set of antigens that is expressed in all GBM patients. Targeting those antigens will introduce an optimized DC-based vaccination therapy that will successfully initiate specific immune responses and eradicate GBM cells.

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